REMARKS

Claims 44-59 are pending and under examination. Claims 44, 47 and 57 have been amended. New claims 60-70 have been added. Support for the amendments can be found throughout the specification and the claims as filed. In particular, support for the amendments can be found in the claims as filed and on page 30, lines 5-7. Support for new claims 60-70 can be found in original claims 9-14. Accordingly, these amendments and new claims do not raise an issue of new matter and entry thereof is respectfully requested.

Regarding the Priority Claim

In the Office Action, it is indicated that the specification lacks the necessary reference to the priority application. It is respectfully pointed out that the priority claim was inserted by amendment in the transmittal referencing the filing of the present application. The first paragraph related to the priority claim has been amended to reflect the status of the parent application, as requested by the Examiner.

Objection to the Claims

The Office Action asserts that claims 52 and 57 are duplicate claims. Without addressing the merits of the objection, Applicant has nevertheless canceled claims 52-56.

Rejections Under 35 U.S.C. § 112, First Paragraph

The rejection of claims 44-59 under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description is respectfully traversed. Applicant respectfully maintains that the specification provides sufficient description and guidance for the claimed methods.

In the Office Action, it is asserted that various generic terminology such as "common ligand," "conserved site," "specificity ligand," "specificity site," "receptor family" and "expansion linker" are recited in the claims but that these terms have broad definitions (Office Action, pp. 3-4). On pages 5-6 of the Office Action, there is again reference to the definition of these terms and that the terms "common ligand" and "specificity ligand" can encompass different moieties. It is respectfully pointed out that none of the terms "conserved site," "specificity ligand," "specificity site," "receptor family" or "expansion linker" are recited in the claims. As for "common ligand," this term is defined in each claim as a "cofactor or analog

thereof." Therefore, the assertion that broad terms are recited in the claims or that adequate support is not provided for these terms is not relevant to the pending claims.

The Office Action further indicates that the "structure of the claimed bi-ligands cannot be determined by the dehydrogenase enzyme or the cofactors" (page 5). Applicant respectfully points out that the claims are directed to methods of identifying a population of bi-ligands to dehydrogenases, not bi-ligands. The Office Action additionally asserts that screening for a bi-ligand that binds to and has specificity for a second dehydrogenase requires further experimentation. However, the claims are directed to methods of identifying a population of bi-ligands and screening for binding activity and therefore require carrying out the steps of the claims. It is unclear how carrying out the steps of a method that may include experiments performing the steps of the method is relevant to written description.

The specification teaches a method for identifying a population of bi-ligands to a receptor (page 15, line 29, to page 16, line 15). The specification further teaches that a receptor can be an enzyme such as a dehydrogenase or an enzyme that binds NAD or NADP (page 11, lines 6-7; page 12, lines 31-33). The specification additionally teaches methods for identifying a bi-ligand to a receptor such as a dehydrogenase or enzyme that binds NAD or NADP (pages 29-46). Therefore, it is respectfully submitted that the specification provides sufficient description and guidance for the claimed methods.

The Office Action indicates on page 6 that adequate disclosure, like enablement, requires representative examples which provide reasonable assurance to one skilled in the art that compounds falling within the scope possess utility and demonstrate that Applicant was in possession of the claimed invention. The Office Action further asserts that "[T]he more unpredictable the art the greater the showing required (e.g. by "representative examples") for both enablement and adequate disclosure." Applicant's representative is not aware of the precedent for such an assertion with respect to written description and would appreciate being provided with the relevant authority so that the case law can be reviewed and responded to appropriately.

With regard to the comment on page 5 of the Office Action that no structure of the identified bi-target ligand is set forth, Applicant respectfully points out that the term "bi-target

ligand" is not recited in the claims. Furthermore, it is respectfully submitted that a number of analogs of NAD and NADP were well known to those skilled in the art. As evidence that a number of such derivatives were well known to those skilled in the art, attached as Exhibit A is a reference by Anderson, "Analogs of Pyridine Nucleotide Coenzymes," The Pyridine Nucleotide Coenzymes, Chap. 4 pp. 91-133, Academic Press, New York (1982). This reference is a review article describing a variety of pyridine nucleotide coenzyme analogs. Furthermore, the specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 U.S.P.Q. 481, 489 (Fed. Cir. 1984).

Applicant maintains that the specification provides sufficient description and guidance to convey to one skilled in the art that Applicant was in possession of the claimed methods for identifying a population of bi-ligands to dehydrogenases or an enzyme that binds NAD or NADP at the time the application was filed. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

The rejection of claims 44-59 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement is respectfully traversed.

Applicant agrees with the assertion in the Office Action on page 8 that compounds that interact with various enzyme targets were known in the art at the time of filing but respectfully disagrees with the assertion that only limited numbers of such compounds were known. Many ligands that bind to enzymes and enzyme cofactor binding sites were well known in the art at the time the application was filed, including enzyme inhibitors, many of which function as drugs for treating various diseases. As evidence that a number of ligands that bind to the cofactor binding site of enzymes were well known to those skilled in the art, attached herewith as Exhibit B is a reference by Radzicka and Wolfenden, Methods Enzymol. 249:284-312 (1995), which describes enzyme inhibitors that bind to various enzyme targets.

Applicants also respectfully disagree with the assertion in the Office Action on page 9 that one skilled in the art would not know how to determine the structure of ligands which are cofactors or cofactor mimics because the determination of the different binding sites in an "enzyme family" would be unpredictable. As discussed above and exemplified in Exhibit A, numerous dehydrogenase cofactor analogs were well known to those skilled in the art. The claims are directed to methods of identifying a population of bi-ligands to dehydrogenases. It is well known to those skilled in the art that dehydrogenases bind to the cofactors NAD and NADP (see page 25, line 30, to page 26, line 1). Clearly, a skilled artisan would readily be able to visualize the structure of an NAD or NADP cofactor or analogs thereof. Moreover, there are hundreds of known structures of dehydrogenases, and therefore the cofactor binding site for dehydrogensease would be readily predictable by one skilled in the art.

Applicant maintains that the specification provides sufficient description and guidance to enable the claimed methods. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

The rejection of claims 44-59 under 35 U.S.C. §, first paragraph, as allegedly containing new matter is respectfully traversed. Applicant respectfully submits that the specification provides sufficient description and guidance for the term "mimic thereof" (see, for example, page 31, lines 23-33). Nevertheless, to further prosecution, the claims have been amended to recite the term "analog." The specification teaches that analogs of cofactors are known (page 30, lines 5-7). Furthermore, as described in Anderson (Exhibit A), numerous NAD or NADP cofactor analogs were well known. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

Rejections Under 35 U.S.C. § 112, Second Paragraph

The rejection of claims 44-59 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite is respectfully traversed. Applicant respectfully maintains that the claims are clear and definite.

Claims 44, 47 and 52 are alleged to be indefinite for the term "mimic thereof." Applicant respectfully submits that this rejection has been rendered moot by deletion of the term "mimic." Accordingly, Applicant respectfully requests that this rejection be withdrawn.

Claims 44-59 are alleged to be indefinite for the phrase "linker has sufficient length and orientation." The specification teaches that a linker provides positioning and orientation of a second ligand (specificity ligand) relative to a common ligand such that they are positioned to bind to their respective substrate and cofactor binding sites (page 10, lines 2-7). This is also illustrated in Figure 1, which shows that the linker bridges the cofactor or analog thereof (common ligand) and second ligand (specificity ligand) to bind simultaneously to the respective cofactor and substrate binding sites (page 5, lines 10-15). Therefore, Applicant respectfully submits that one skilled in the art, based on the teachings in the specification and the language of the claim that "a linker has sufficient length and orientation to direct a second ligand to a substrate binding site," would understand that the claims are clear and definite. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

Claims 44-59 are alleged to be indefinite and to be incomplete for the recitation of step (e). Claims 44, 47 and 57 have been amended to recite separate steps for screening and identifying a bi-ligand that binds to and has specificity for a first dehydrogenase (steps (c) and (d)) and a bi-ligand that binds to and has specificity for a second dehydrogenase (steps (e) and (f)). Accordingly, Applicant submits that these claims are clear and definite and respectfully requests that these rejections be withdrawn.

Rejection Under 35 U.S.C. § 101

The rejection of claims 44-59 under 35 U.S.C. § 101 and under 35 U.S.C. § 112, first paragraph, as allegedly lacking utility is respectfully traversed. Applicant respectfully submits that the claimed methods have specific and substantial utility.

The specification provides sufficient description and guidance to support a specific and substantial utility. The methods are directed to generating populations or libraries of compounds focused to have specificity for binding to particular receptor or enzyme families (page 6, line 19, to page 7, line 27; page 43, line 28, to page 44, line 9; page 47, lines 7-15). The specification further teaches that the methods can be used to generate libraries for screening therapeutic agents that target various diseases, for example, targeting a pathogen or targeting cancer cells (page 17, lines 4-19). Clearly, the advantageous methods for generating a population or library of compounds that allow the same population or library to be screened against multiple targets

which bind a common ligand has specific and substantial utility. The fact that billions of dollars are spent in the pharmaceutical industry to create and screen compound libraries further substantiates the utility of methods that advantageously generate libraries with desirable properties.

The Office Action repeatedly asserts that the further experimentation must be performed to determine if the bi-ligands have activity and that this therefore results in lack of specificity of the asserted utility. Applicant draws the Examiner's attention to MPEP 2107, directed to utility. MPEP § 2107.1 describes examples that meet and do not meet the criteria of specific and substantial utility. The MPEP exemplifies lack of specific utility, for example, for a compound that may be useful in treating unspecified disorders or is merely described as having "useful biological" properties. However, as discussed above and disclosed in the specification, the claimed methods are used to generate compounds that can be screened for specific therapeutic applications. Accordingly, the assertion that the claimed methods do not have specific utility is clearly not supported by the guidelines on specific utility set forth in the MPEP. With regard to substantial utility, MPEP 2107.01 clearly indicates, contrary to the assertion in the Office Action, that "an assay method for identifying compounds that themselves have a 'substantial utility' define a 'real world' context of use," in contrast to methods of making a material that itself has no specific, substantial and credible utility. To the contrary, the claimed methods are directed to generating compounds that are used to screen for drug leads, clearly a specific, substantial and credible utility.

Moreover, MPEP 2017.01 admonishes Office personnel not to interpret the phrase "immediate benefit to the public" to mean that products or services based on the claimed invention must be "currently available to the public in order to satisfy the utility requirement. "Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility." Clearly such an admonishment is not being followed based on the reasoning set forth in the Office Action. Furthermore, the description in the Office Action regarding "research tools" is irrelevant since the claims are clearly not directed to research tools but to methods that are useful for drug discovery.

Applicant respectfully submits that the assertion in the Office Action that the claimed methods of generating populations and libraries of compounds for identifying drug candidates lack utility is clearly without merit. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

Double Patenting

Claims 44-59 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting as allegedly unpatentable over claims 1-28 of copending application serial No. 10/103,535. Applicant respectfully requests that this rejection be held in abeyance until there is an indication of allowable subject matter.

In light of the amendments and remarks herein, Applicant submits that the claims are now in condition for allowance and respectfully requests a notice to this effect. The Examiner is invited to call the undersigned agent if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

McDERMOTT WILL & EMERY LLP

Deborah L. Cadena
Registration No. 44,048

4370 La Jolla Village Drive, Suite 700
San Diego, CA 92122
Phone: 858.535.9001 DLC:jrl
Facsimile: 858.597.1585

Please recognize our Customer No. 41552 as our correspondence address.

Date: February 8, 2005

SDO 26001-1.066692.0024

The Pyridine Nucleotide Coenzymes

Edited by

JOHANNES EVERSE

Department of Biochemistry
Texas Tech University
Health Sciences Center
Lubbock, Texas

BRUCE ANDERSON

Department of Biochemistry
Virginia Polytechnic Institute
and State University
Blacksburg, Virginia

KWAN-SA YOU

Department of Pediatrics
Duke University Medical Center
Durham, North Carolina

1982

ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

New York London

Paris San Diego San Francisco São Paulo Sydney Tokyo Toronto

aplan

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data
Main entry under title:

The Pyridine nucleotide coenzymes.

Includes index.
1. Coenzymes. 2. Pyridine. 3. Nucleotides.
I. Everse, Johannes. II. Anderson, Bruce.
III. You, Kwan-sa. [DNLM: 1. NAD. 2. NADP.
QU 163 V217]
QP601.P95 574.19'25 81-17632
ISBN 0-12-244750-6 AACR2

PRINTED IN THE UNITED STATES OF AMERICA

82 83 84 85 9 8 7 6 5 4 3 2 1

Co

List of Contributors
Preface
Nathan O. Kaplan: A Tribute by M
To Nate by Fritz Lipmann
To Nate by W. D. McElroy
Origins of Pyridine Nucleotide Rese
McCollum-Pratt Institute in the J

1 Evolution of Coenzymes a Nucleotides

Harold B. White III

I. Catalysts in Prebiotic E
II. Existence of Coenzyme Apparatus Evolved
III. Proteins as a Secondary Coenzyme-Dependent I
IV. Coenzyme Requiremen
V. Nucleotide Structure of Evolution
VI. Vestiges of a Polynucl Biochemistry
VII. Amino Acids as an Ex
VIII. Implications for Enzym
IX. Difficulties with the M
X. Some Considerations o Coenzymes
References

4

Analogs of Pyridine Nucleotide Coenzymes

BRUCE M. ANDERSON

I.	Introduction	91
II.	Synthesis of Coenzyme Analogs	92
	A. Chemical Methods	92
	B. Enzymatic Methods	94
III.	Specific Modifications of NAD	98
IV.	Properties of Coenzyme Analogs	107
	A. Spectral Properties	107
	B. Oxidation-Reduction Properties	109
V.	Applications	110
	A. Enzymatic Reactions	110
	B. Site-Labeling Studies	116
	C. *In Vivo* Studies	120
	D. Clinical Studies	122
	E. Evolution of Dehydrogenases	123
VI.	Concluding Remarks	124
	References	126

I. INTRODUCTION

The biological functioning of the pyridine nucleotide coenzymes, NAD and NADP, can be related to the unique combination of functional groups that compose these molecules. In a simplistic approach, one considers these compounds to be comprised of a pyridine base nicotinamide, a purine base adenine, two ribose moieties, a pyrophosphate grouping and in the case of NADP, an additional phosphoryl group. In the functioning of these compounds in oxidation–reduction reactions, hydride transfer to the nicotinamide moiety to

THE PYRIDINE NUCLEOTIDE COENZYMES

ISBN 0-12-244750-6

produce the 1,4-dihydronicotinamide ring system of the reduced coenzymes is well established and provides adequate documentation of the importance of this functionality. The components of the remaining portions of the coenzyme molecules, adenosine diphosphoribose (ADPR) and adenosine diphosphoribose phosphate can be considered important in the selective interactions of these molecules with enzymes. Such interactions can play a role in the specific orientation of the coenzymes as related to bound substrate and enzyme catalytic groups. In the case of the adenine moiety, intramolecular interactions can serve through energy transfer to activate the nicotinamide ring for subsequent hydride transfer steps. In considering enzyme-catalyzed oxidation–reduction reactions, one views the two coenzymes as 3-carboxamidopyridinium derivatives containing either ADPR or phospho-ADPR, emphasizing a greater importance for the oxidation–reduction-active nicotinamide ring. However, with the more recently established importance for NAD in ADP-ribosylation reactions, a quite different viewpoint emerges in which one considers the molecule to be composed of an important ADPR moiety attached to an effective leaving group, the nicotinamide ring. It can be anticipated that the different components of the pyridine nucleotides would play different roles in these two different modes of functioning. Whether or not the biological functioning involves oxidation–reduction or ADP-ribosylation, changes in the structure of the various components of these molecules can have pronounced effects on the reactions catalyzed, and the modification of these components to produce structural analogs has provided an effective experimental approach for the study of the mode of action of these molecules. Analogs of the pyridine nucleotide coenzymes have played an important role in the elucidation of the mechanism of action of many pyridine nucleotide-requiring enzymes. The following discussion of pyridine nucleotide analogs has been limited for the most part to the consideration of NAD analogs since the preponderance of studies in this area relate to this coenzyme. However, it should be noted that many aspects of the following discussion are equally applicable to the phosphorylated coenzyme, NADP.

II. SYNTHESIS OF COENZYME ANALOGS

A. Chemical Methods

Both chemical and enzymatic methods have been used for the synthesis of coenzyme analogs in which one or more moieties of the dinucleotide molecule have been modified. The earliest reported coenzyme analog was nicotinamide hypoxanthine dinucleotide which was prepared chemically by Schlenk *et al.* [1] in 1938 through the treatment of NAD with nitrous acid. It was later shown by Kaplan *et al.* [2] that the same compound could also be prepared through

enzymatic deamination. A number of c
through the chemical condensation of n
densing agents such as *N,N*-dicyclohexyl
anhydride [5]. Such condensations can l
product plus symmetrical by-products
exchange chromatography. The formatic
employing the method described by Moff
nucleoside monophosphates is converted
and then condensed with the second, des
these condensation techniques, Woenckh
impressive array of coenzyme analogs,
tailed in a recent review [4]. Methods are
synthesis of NAD and NAD analogs fr
ribose derivatives [6–12].

Several coenzyme analogs have been p
tion of NAD or other NAD analogs. This
by the lability of the pyridine nucleotide
preparation of several very interesting an
hypoxanthine dinucleotide through nitro
been noted [1]. Other direct chemical n
NAD include: (1) reaction with ethyl
hydroxyethyl derivatives [13]; (2) react
carboxymethyl-NAD which was further
form N^6-[*N*-(6-aminohexyl)acetamide]-
water to form the 8-bromo derivative
dehyde to form the 1,N^6-ethenoadenine
propiolactone to form the N^1- and N^6-2-

Direct chemical modification of the p
led to the successful formation of coenzy
reaction with NAD was used to produce
[20] which could be diazotized with n
dinucleotide [20,21]. 3-Diazopyridine
cuprous chloride, cuprous bromide an
3-chloropyridine, 3-bromopyridine, anc
[22]. Diazotized 3-aminopyridine aden
with several sulfhydryl compounds to p
however, the resulting pyridine nucleoti
terized [21]. Thionicotinamide adenine d
3-cyanopyridine derivative by treatment
pyridine adenine dinucleotide [25] was
to 3-chloroacetylpyridine adenine dinu
adenine dinucleotide was prepared from

enzymatic deamination. A number of coenzyme analogs have been prepared through the chemical condensation of nucleoside monophosphates using condensing agents such as *N,N*-dicyclohexylcarbodiimide [3,4] and trifluoroacetic anhydride [5]. Such condensations can lead to mixtures containing the desired product plus symmetrical by-products which can be separated through ion-exchange chromatography. The formation of by-products can be avoided by employing the method described by Moffat and Khorana [6] in which one of the nucleoside monophosphates is converted to the phosphomorpholidate derivative and then condensed with the second, desired nucleoside monophosphate. Using these condensation techniques, Woenckhaus and colleagues have synthesized an impressive array of coenzyme analogs, the properties of which have been detailed in a recent review [4]. Methods are also available for the complete *de novo* synthesis of NAD and NAD analogs from the appropriate nitrogen bases and ribose derivatives [6–12].

Several coenzyme analogs have been prepared through the chemical modification of NAD or other NAD analogs. This route of synthesis is somewhat limited by the lability of the pyridine nucleotide molecule but has, however, led to the preparation of several very interesting analogs. The preparation of nicotinamide hypoxanthine dinucleotide through nitrous acid treatment of NAD has already been noted [1]. Other direct chemical modifications of the adenine moiety of NAD include: (1) reaction with ethylene oxide to form the N^1- and N^6-hydroxyethyl derivatives [13]; (2) reaction with iodoacetic acid to form N^6-carboxymethyl-NAD which was further condensed with 1,6-diaminohexane to form N^6-[*N*-(6-aminohexyl)acetamide]-NAD [14]; (3) reaction with bromine water to form the 8-bromo derivative [15–17]; (4) reaction with chloracetaldehyde to form the 1,N^6-ethenoadenine derivative [18]; and (5) reaction with propiolactone to form the N^1- and N^6-2-carboxyethyl derivatives [19].

Direct chemical modification of the pyridinium moiety of NAD has likewise led to the successful formation of coenzyme analogs. The Hofmann hypobromite reaction with NAD was used to produce 3-aminopyridine adenine dinucleotide [20] which could be diazotized with nitrous acid to 3-diazopyridine adenine dinucleotide [20,21]. 3-Diazopyridine adenine dinucleotide was reacted with cuprous chloride, cuprous bromide and potassium iodide to synthesize the 3-chloropyridine, 3-bromopyridine, and 3-iodopyridine analogs, respectively [22]. Diazotized 3-aminopyridine adenine dinucleotide was reported to react with several sulfhydryl compounds to produce 3-pyridyl thioether derivatives; however, the resulting pyridine nucleotide analogs were not isolated and characterized [21]. Thionicotinamide adenine dinucleotide [23] can be converted to the 3-cyanopyridine derivative by treatment with silver nitrate [24]. 3-Diazoacetylpyridine adenine dinucleotide [25] was converted by reaction with LiCl–HCl to 3-chloroacetylpyridine adenine dinucleotide [25]. Tetrahydronicotinamide adenine dinucleotide was prepared from NADH by catalytic reduction [26]. The

spin-labeled coenzyme analog, 3-(4',4',5',5'-tetramethyl-3'-oxide-1'-oxyl-2'-imidazolinyl)pyridine adenine dinucleotide was prepared by the reaction of pyridine-3-aldehyde adenine dinucleotide [27,28] with 2,3-dihydroxyaminobutane, followed by oxidation with lead dioxide [29].

In addition to specific modification of the pyridine and adenine moieties of pyridine nucleotides, a number of new derivatives have been prepared through the chemical modification of other existing analogs. In this respect, nicotinamide 6-thiopurine dinucleotide [30] was reacted with methylmercuric chloride to form nicotinamide *S*-methylmercuric thioinosine dinucleotide [31]. Nicotinamide 5-acetyl-4-methylimidazole dinucleotide was reacted with bromine to produce nicotinamide 5-bromoacetyl-4-imidazole dinucleotide [32].

There are a few examples of direct chemical modification of the ribose moieties of pyridine nucleotides. The adenine ribose of NAD has been modified by carbodiimide condensation of arylazido-β-alanine with NAD to form the arylazido-β-alanyl ester of NAD [33]. The nicotinamide ribose of NADP has been oxidized through periodate oxidation to form the 2',3'-dialdehyde which could be isolated by alcohol precipitation of the barium salt [34].

B. Enzymatic Methods

The major enzymatic method used for the preparation of pyridine nucleotide coenzyme analogs involves the transglycosidase activity of mammalian NAD glycohydrolases (NADases). This enzyme-catalyzed transglycosidation reaction, frequently referred to as the pyridine base-exchange reaction, has historical significance since it was the preferred method of synthesis in the early studies of coenzyme analogs. Early studies [35,36] of calf spleen NADase-catalyzed hydrolysis of NAD suggested the reaction to proceed through the release of nicotinamide and the formation of an ADPR-enzyme intermediate as shown in Fig. 1. The attack of water on the uncharacterized ADPR-enzyme intermediate completes the hydrolytic reaction with the formation of free enzyme and ADPR. Noncompetitive inhibition by nicotinamide in this reaction was observed to involve a competition between nicotinamide and water for the ADPR-enzyme intermediate leading to the reformation of NAD. If the reaction is carried out by incubating NAD, enzyme and ^{14}C-labeled nicotinamide, ^{14}C-labeled NAD is formed [35,36]. Simple reversal of the reaction was ruled out since no labeled NAD could be produced by incubating the enzyme with ADPR and ^{14}C-labeled nicotinamide. These observations suggested the possibility that other pyridine bases might also compete with water for the ADPR–enzyme intermediate, thus producing pyridine nucleotides containing pyridine bases other than nicotinamide. This was quickly verified by the demonstration that isonicotinic acid hydrazide functions in this transglycosidation reaction to form isonicotinic acid hydrazide adenine dinucleotide [37,38]. Pig brain NADase was also ob-

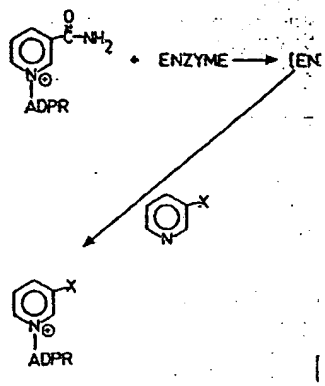


Fig. 1. Reactions catalyz

served to catalyze the transglycosidatio
first pyridine-substituted coenzyme a
reactions, 3-acetylpyridine adenine di
mammalian NADase-catalyzed transgly
by the fact that the majority of the substi
analogs reported were prepared throu
have been recognized to be important i
reaction for the preparation of coenzym
the pyridine base to be exchanged and
attempts to exchange nitrogen bases c
cleotide form have been unsuccessful,
volved as evidenced by the higher yi
showing a closer structural analogy to r
reaction requires a competition with
nitrogen basicity [40] or limited water
ineffective in promoting analog forma
effectiveness of pyridine bases in the
pyridine is considerably less effective t
tion of 6-aminonicotinamide adenine d
culty leading to very low yields of thi

The two enzymes most frequently
nucleotide analogs through the pyridi
NADase and pig brain NADase. Both
for the most part, crude preparations o
processes. The membraneous environr

derson

',5'-tetramethyl-3'-oxide-1'-oxyl-2'-
: was prepared by the reaction of
[27,28] with 2,3-dihydroxyamino-
oxide [29].
he pyridine and adenine moieties of
ivatives have been prepared through
analogs. In this respect, nicotinamide
with methylmercuric chloride to form
e dinucleotide [31]. Nicotinamide
as reacted with bromine to produce
nucleotide [32].
hemical modification of the ribose
ne ribose of NAD has been modified
o-β-alanine with NAD to form the
: nicotinamide ribose of NADP has
to form the 2',3'-dialdehyde which
f the barium salt [34].

e preparation of pyridine nucleotide
sidase activity of mammalian NAD
atalyzed transglycosidation reaction,
xchange reaction, has historical sig-
l of synthesis in the early studies of
f calf spleen NADase-catalyzed hy-
to proceed through the release of
R–enzyme intermediate as shown in
terized ADPR–enzyme intermediate
ormation of free enzyme and ADPR.
in this reaction was observed to in-
and water for the ADPR–enzyme
AD. If the reaction is carried out by
nicotinamide, ^{14}C-labeled NAD is
tion was ruled out since no labeled
enzyme with ADPR and ^{14}C-labeled
l the possibility that other pyridine
ADPR–enzyme intermediate, thus
ing pyridine bases other than
the demonstration that isonicotinic
idation reaction to form isonicotinic
]. Pig brain NADase was also ob-

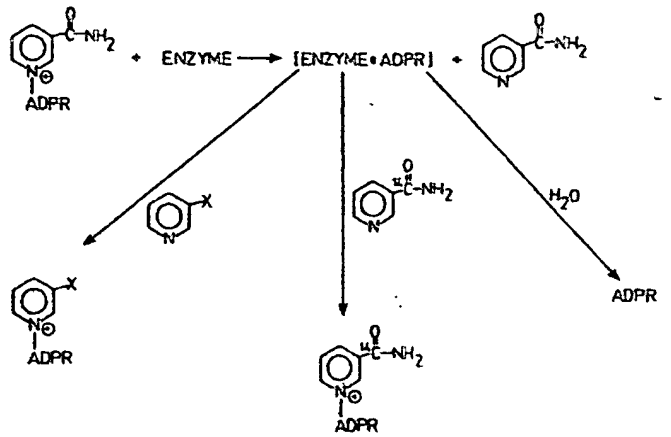


Fig. 1. Reactions catalyzed by NAD glycohydrolases.

served to catalyze the transglycosidation and was used for the preparation of the first pyridine-substituted coenzyme analog that functioned in dehydrogenase reactions, 3-acetylpyridine adenine dinucleotide [39]. The importance of the mammalian NADase-catalyzed transglycosidation reaction is readily documented by the fact that the majority of the substituted pyridine and substituted nicotinamide analogs reported were prepared through this synthetic route. Several factors have been recognized to be important in the application of the transglycosidation reaction for the preparation of coenzyme analogs. Selective interactions between the pyridine base to be exchanged and an enzyme site appear necessary, since attempts to exchange nitrogen bases other than pyridine derivatives into dinucleotide form have been unsuccessful. Selective binding processes may be involved as evidenced by the higher yields of analog obtained with those bases showing a closer structural analogy to nicotinamide. Since the transglycosidation reaction requires a competition with water, pyridine derivatives with low ring nitrogen basicity [40] or limited water solubility can be expected to be relatively ineffective in promoting analog formation. Steric factors can also diminish the effectiveness of pyridine bases in the transglycosidation reaction. 3-Benzoylpyridine is considerably less effective than 3-acetylpyridine [23], and the formation of 6-aminonicotinamide adenine dinucleotide proceeds only with great difficulty leading to very low yields of this analog [41,42].

The two enzymes most frequently employed for the preparation of pyridine nucleotide analogs through the pyridine base exchange reaction are calf spleen NADase and pig brain NADase. Both of these enzymes are membrane bound and for the most part, crude preparations of these enzymes were used in the synthetic processes. The membraneous environment of these enzymes was observed not to

be essential for transglycosidase activity since a number of mammalian NADases have been solubilized and demonstrated to retain this activity [43–47]. In these studies, NADases purified to high specific activities were demonstrated to still effectively catalyze the pyridine base-exchange reaction. Mechanistic studies of mammalian NADases have been hampered by the lability of solubilized and purified forms of the enzyme. Substrate specificity studies of mammalian NADases [44,45,48–50] have, however, demonstrated that a number of pyridine nucleotide analogs prepared through the transglycosidation reaction also serve as substrates in the hydrolytic reactions catalyzed by these enzymes. Therefore, in those cases where the analog being formed is also a substrate for the enzyme, prolonged incubation with the enzyme can serve to decrease the overall yield of the desired analog. This is especially true in the preparation of 3-acetylpyridine adenine dinucleotide as well as several other analogs having a close structural similarity to the natural substrate, NAD. In order to maximize the yield of analog formed in a transglycosidation reaction, it is advantageous to monitor the loss of NAD and the appearance of analog. Such analyses have been successful when the analog being formed possesses unique properties differing from those of NAD. For example, 3-acetylpyridine adenine dinucleotide functions as a coenzyme with yeast alcohol dehydrogenase and the reduced analog has an absorption maximum at 365 nm which differs from the 340 nm maximum characteristic of NADH. Therefore, the enzymatic conversion of NAD to the 3-acetylpyridine analog can be monitored spectrophotometrically through the yeast alcohol dehydrogenase assay of samples from the incubation mixture. As shown in Fig. 2 (line 1) [51], the 365–340 ratio should increase from an initial value of approximately 0.6 and approach the theoretical value of 1.4 for pure 3-acetylpyridine analog. The reaction can be terminated when the assay indicates that further incubation causes a decrease in the presence of analog (Fig. 2, line 3). This method can be used to determine whether or not a given NADase possesses transglycosidase activity. Bovine seminal fluid NADase purified to homogeneity [52] does not catalyze the transglycosidation reaction with 3-acetylpyridine [53], and as expected, no change in the 365/340 ratio is observed upon incubation of NAD and 3-acetylpyridine with this enzyme (Fig. 2, line 2).

In those cases where the pyridine nucleotide analog to be formed does not function as a coenzyme in dehydrogenase-catalyzed reactions, a less effective method to monitor the reaction must be considered. The formation of the 3-aminopyridine analogs of NAD and NADP can be monitored by measuring the 330 nm absorption characteristic of pyridinium derivatives of 3-aminopyridine [54]. In a similar manner, the formation of isonicotinic acid hydrazide adenine dinucleotide can be followed by measuring the appearance of 385 nm absorbance of the analog under alkaline conditions [37,38]. If the analog has no distinguishing properties, one may be limited to monitoring only the disappearance of NAD. In most cases where the analog does not function as a coenzyme in dehydrogenase reactions, it also does not fun
hydrolysis and therefore monitoring of

Not all mammalian NADases cata
bovine seminal fluid NADase has alr
seminal fluid enzyme differs in many
[55] in that it is a soluble, extracellul
mammalian NADases to catalyze the
related to whether or not the enzyme is
erythrocyte NADase which is membra
membrane lipid phase transitions, doe
tion with 3-acetylpyridine or ^{14}C-labe
teresting NADases have been observed
of which have been demonstrated to ca
pyridine bases to form pyridine nuc
Bungarus fasciatus venom has been
greater stability than that observed w

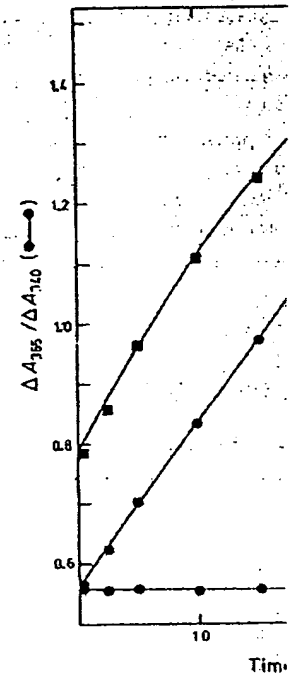


Fig. 2. A comparison of the involvement of
NADase and bull semen NADase. See text for

nce a number of mammalian NADases
) retain this activity [43–47]. In these
c activities were demonstrated to still
ange reaction. Mechanistic studies of
ed by the lability of solubilized and
e specificity studies of mammalian
lemonstrated that a number of pyridine
nsglycosidation reaction also serve as
yzed by these enzymes. Therefore, in
:d is also a substrate for the enzyme,
serve to decrease the overall yield of
in the preparation of 3-acetylpyridine
her analogs having a close structural
order to maximize the yield of analog
is advantageous to monitor the loss of
analyses have been successful when
:e properties differing from those of
ine dinucleotide functions as a coen-
l the reduced analog has an absorption
ie 340 nm maximum characteristic of
sion of NAD to the 3-acetylpyridine
ically through the yeast alcohol dehy-
tion mixture. As shown in Fig. 2 (line
rom an initial value of approximately
1.4 for pure 3-acetylpyridine analog.
ssay indicates that further incubation
; (Fig. 2, line 3). This method can be
NADase possesses transglycosidase
rified to homogeneity [52] does not
th 3-acetylpyridine [53], and as ex-
)served upon incubation of NAD and
line 2).
:otide analog to be formed does not
-catalyzed reactions, a less effective
considered. The formation of the
P can be monitored by measuring the
)ium derivatives of 3-aminopyridine
f isonicotinic acid hydrazide adenine
he appearance of 385 nm absorbance
38]. If the analog has no distinguish-
ring only the disappearance of NAD.
: function as a coenzyme in dehy-

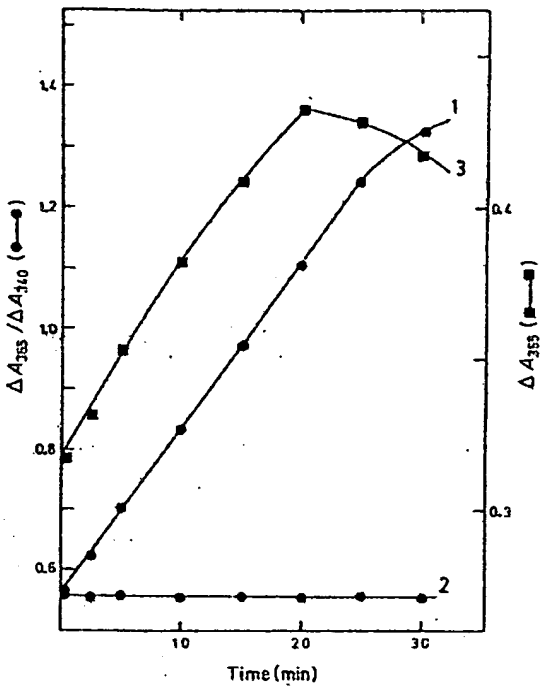


Fig. 2. A comparison of the involvement of 3-acetylpyridine in reactions catalyzed by pig brain NADase and bull semen NADase. See text for explanation. From Yost and Anderson [51].

drogenase reactions, it also does not function as a substrate for NADase-catalyzed hydrolysis and therefore monitoring of the analog formation is less important.

Not all mammalian NADases catalyze a transglycosidation reaction. The bovine seminal fluid NADase has already been noted as one exception. The seminal fluid enzyme differs in many respects from other mammalian NADases [55] in that it is a soluble, extracellular glycoprotein. However, the ability of mammalian NADases to catalyze the transglycosidation reaction is not strictly related to whether or not the enzyme is soluble or membrane bound. The bovine erythrocyte NADase which is membrane bound and significantly influenced by membrane lipid phase transitions, does not catalyze the transglycosidation reaction with 3-acetylpyridine or ^{14}C-labeled nicotinamide [56]. A number of interesting NADases have been observed in various snake venoms [57–59], several of which have been demonstrated to catalyze the transglycosidation reaction with pyridine bases to form pyridine nucleotide analogs [58]. The NADase from *Bungarus fasciatus* venom has been extensively purified [51], and due to a greater stability than that observed with purified mammalian NADases, should

provide an effective system to study the mechanism of transglycosidase activity of NADases.

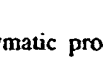
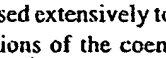
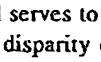
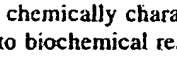
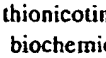
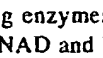
A second and less frequently employed enzymatic method for the preparation of pyridine nucleotide analogs involves the condensation of pyridine mononucleotides with purine riboside triphosphates as catalyzed by NAD pyrophosphorylase [60]. Due to the specificity of this enzyme [61], this method has been applied predominantly for reactions of nicotinamide mononucleotide with nucleoside triphosphates containing nonpolar heterocyclic bases other than adenine. Several adenine-substituted analogs of NAD have been prepared using this procedure [61-63].

III. SPECIFIC MODIFICATIONS OF NAD

The chemical and enzymatic procedures for the synthesis of pyridine nucleotide analogs have been used extensively to prepare analogs of NAD containing alterations in all major portions of the coenzyme molecule. Table I provides a listing of NAD analogs subdivided according to alterations in different parts of the coenzyme molecule and serves to exemplify the diversity of derivatives that have been prepared. Some disparity exists in the degree to which some of the derivatives listed have been chemically characterized. Those compounds showing interesting applications to biochemical research have been more extensively purified and most effectively characterized. Some, such as 3-acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide have become essentially commonplace biochemicals and are commercially available. A number of other analogs with obvious applications to biochemical research have more recently become available commercially, a factor which has a profound effect on the studies of these compounds. A variety of reasons can be cited for the preparation of the analogs listed in Table I. Some of the derivatives were to be used to study interactions at the coenzyme binding sites of dehydrogenases. Others were designed to investigate functional group involvement in hydride transfer reactions. Those derivatives containing spin-labeling groups, fluorophores and site-labeling groups have obvious applications for the study of pyridine nucleotide-requiring enzymes. Excluded from Table I are the isotopically labeled derivatives of NAD and NAD analogs. A sampling of a number of excellent studies can be cited [156-177] to provide information concerning the preparation and properties of isotopically labeled derivatives. Although Table I lists only analogs of NAD, in several cases where the analog has exhibited properties of importance to biochemical studies, the corresponding derivative of NADP has been prepared. In one case, the preparation of selenonicotinamide adenine dinucleotide phosphate was reported [178] while the corresponding NAD analog was not.

TABLE I

Analogs of NAD

Compound number	Substituent
	I. Alterations in the nicotinam
	A. Alterations in the pyrid
	1. Substituted pyridin
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
15	
16	

nechanism of transglycosidase activity

enzymatic method for the preparation ves the condensation of pyridine triphosphates as catalyzed by NAD ficity of this enzyme [61]. this method ions of nicotinamide mononucleotide nonpolar heterocyclic bases other than ogs of NAD have been prepared using

D

res for the synthesis of pyridine nu- to prepare analogs of NAD containing enzyme molecule. Table I provides a ling to alterations in different parts of mplify the diversity of derivatives that s in the degree to which some of the naracterized. Those compounds show- l research have been more extensively zed. Some, such as 3-acetylpyridine le adenine dinucleotide have become and are commercially available. A lications to biochemical research have cially, a factor which has a profound A variety of reasons can be cited for the I. Some of the derivatives were to be me binding sites of dehydrogenases. ctional group involvement in hydride containing spin-labeling groups, e obvious applications for the study of xcluded from Table I are the isotopi- D analogs. A sampling of a number of to provide information concerning the labeled derivatives. Although Table I ases where the analog has exhibited udies, the corresponding derivative of he preparation of selenonicotinamide orted [178] while the corresponding

TABLE I

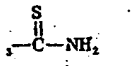
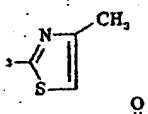
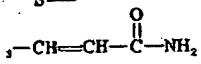
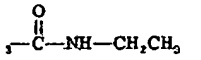
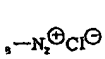
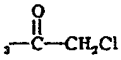
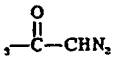
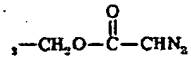
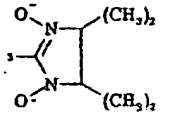
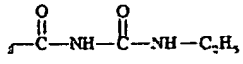
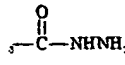
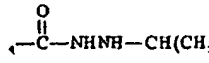
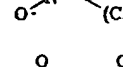
Analogs of NAD

Compound number	Substituent	Reference	Enzymatically active
	I. Alterations in the nicotinamide mononucleotide moiety		
	A. Alterations in the pyridine ring		
	1. Substituted pyridine		
1	$_3$—H	*27*	No
2	$_3$—CH(=O)	*27,28*	Yes
3	$_3$—C(=O)—CH_3	*27,28*	Yes
4	$_3$—C(=O)—$CH(CH_3)_2$	*23,64*	Yes
5	$_3$—C(=O)—CH_2CH_3	*25,65*	Yes
6	$_3$—C(=O)—$CH_2CH_2CH_3$	*65*	Yes
7	$_3$—C(=O)—C_6H_5	*23,64*	Yes
8	$_3$—C(=O)OH	*66*	No
9	$_3$—C(=O)—OC_2H_5	*66*	No
10	$_3$—C(=O)—NHOH	*23,64*	Yes
11	$_3$—C(=O)—$NHNH_2$	*23,64*	Yes
12	$_3$—CH_3	*27*	No
13	$_3$—CHOH—CH_3	*27*	No
14	$_3$—CH=NOH	*23,64,67*	Yes
15	$_3$—NH_2	*20,21,23,40,64*	No
16	$_3$—NH—C(=O)—CH_3	*23,64*	No

(continued)

TABLE I (*Continued*)

Analogs of NAD

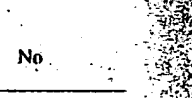
Compound number	Substituent	Reference	Enzymatically active
17		*23,64,68,69*	Yes
18		*70*	No
19		*23,64*	No
20		*27*	No
21		*20,21*	No
22		*25,71*	Yes
23		*25*	—
24		*26*	Yes
25		*22*	Yes
26		*22*	Yes
27		*22,72*	Yes
28		*73*	No
29		*74*	—
30		*29*	—
31		*75*	No
32		*37*	No
33		*37*	No
34			
35			
36	Nicotine		
37	Cotinine		
38			
39	N^1-2-(Lactyl)-N^2-(Isonicotinoyl) hydrazine		
40	N^1-(Isonicotinoyl)-N^2-4-(2, 2, 6, 6-tetramethyl-4-hydroxypiperidine-1-oxyl) hydrazine		
41			
42			
43			
44			
45			
46			
47			
48			

2. Substituted nicotinamide

49			

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent	Reference	Enzymatically active
34		*25*	No
35		*25*	No
36	Nicotine	*76,77*	No
37	Cotinine	*78*	No
38		*79,80*	No
39	N^1-2-(Lactyl)-N^2-(Isonicotinoyl) hydrazine	*81*	No
40	N^1-(Isonicotinoyl)-N^2-4-(2, 2, 6, 6-tetramethyl-4-hydroxypiperidine-1-oxyl) hydrazine	*81*	No
41		*82*	—
42		*82*	—
43		*83*	—
44		*83*	—
45		*84*	—
46		*84*	—
47		*85*	—
48		*86*	No
2. Substituted nicotinamide			
49		*87*	No

(*continued*)

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent	Reference	Enzymatically active
50	$_4$—CHOH—C(=O)—CH_2OH	*88,89*	No
51	$_4$—CH_2—C(=O)—CH_3	*88,89*	No
52	$_4$—OCH_3	*90*	No
53	$_4$—CH_2—CHOH—C(=O)H	*88,89*	No
54	$_4$—CH_3	*40,91*	No
55	$_5$—CH_3	*40,65,91*	No
56	$_5$—NH_2	*40*	No
57	$_6$—NH_2	*41,92*	No
58	(3*S*)-5-(3-Carboxy-3-hydroxypropyl)	*93-95*	Yes
59	(3*R*)-5-(3-Carboxy-3-hydroxypropyl)	*93-95*	No
60	$_5$—carboxy	*96*	No
	3. Disubstituted pyridine		
61	$_3$—CN, $_4$—CH_3	*91*	No
62	$_3$—CN, $_5$—CH_3	*91*	No
63	$_3$—C(=S)—NH_2, $_5$—CH_3	*91*	No
64	$_3$—C(=S)—NH_2, $_5$—CH_3	*91*	No
65	$_3$—C(=O)—CH_3, $_5$—CH_3	*97*	—
	B. Replacement of the pyridine ring		
66	1,4,5,6-Tetrahydronicotinamide	*98*	No
67	4-Amino-5-carboxamideimidazole	*99*	No
68	5-(β-Ethylamino)imidazole (histamine)	*100*	No
69	Imidazole	*101*	No
70	5-(2-Amino-2-carboxyethyl)imidazole (histidine)	*101*	No
71	5-(2-Acetylaminoethyl)imidazole (acetylhistamine)	*101*	No

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent
72	2-Amino-1,3,4-Thiadiazole
73	2-(β-Ethylamino)thiadiazole
74	4-Methyl-5-(β-hydroxyethyl)thia
75	Benzimidazole
76	Hypoxanthine
77	Adenine
78	3-Carboxamido-4-keto-1,4-dihy (4-pyridone)
79	2- and 6-Pyridones: 3-carboxamido-2-keto-1,2-dih pyridine
80	Benzene
81	2-Methyl-3-hydroxyethyl-4-nitr (Flagl)
	C. Replacement of the ribose moiety
82	Pentanol
83	Dideoxyribose
84	Glucose
	II. Alterations in the adenylic acid moiety
	A. Substituted adenine
85	N^6-(2-hydroxyethyl)
86	N^1-(2-hydroxyethyl)
87	N^1-oxide
88	N^1-(2-carboxyethyl)
89	N^6-(2-carboxyethyl)
90	N^6-[*N*-(*N*-acryloyl-1-methoxy aminopentyl)propionamide]
91	N^6-(6-aminohexyl)
92	N^6-(carboxymethyl)
93	N^6-[*N*-(6-aminohexyl)acetami
94	8-Br
95	8-(6-aminohexyl)amino
96	8-SH
97	1,N^6-etheno
98	N^6-(2,2,6,6-tetramethylpiperi
99	N^1-aminoethyl
100	N^6-aminoethyl
101	N^1-CH_2—CH(OH)—CH_2OH
102	N^6-CH_2—CH(OH)—CH_2OH

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent	Reference	Enzymatically active
72	2-Amino-1,3,4-Thiadiazole	*102*	No
73	2-(β-Ethylamino)thiadiazole	*103*	No
74	4-Methyl-5-(β-hydroxyethyl)thiazole	*104*	No
75	Benzimidazole	*101*	No
76	Hypoxanthine	*101*	No
77	Adenine	*5*	No
78	3-Carboxamido-4-keto-1,4-dihydropyridine (4-pyridone)	*105*	No
79	2- and 6-Pyridones: 3-carboxamido-2-keto-1,2-dihydro-pyridine	*106*	No
80	Benzene	*107*	No
81	2-Methyl-3-hydroxyethyl-4-nitroimidazole (Flagl)	*108*	No
	C. Replacement of the ribose moiety		
82	Pentanol	*109*	No
83	Dideoxyribose	*110*	Yes
84	Glucose	*111*	No
	II. Alterations in the adenylic acid moiety		
	A. Substituted adenine		
85	N^6-(2-hydroxyethyl)	*13*	Yes
86	N^1-(2-hydroxyethyl)	*13*	Yes
87	N^1-oxide	*112*	*Yes*
88	N^1-(2-carboxyethyl)	*19*	Yes
89	N^6-(2-carboxyethyl)	*19*	Yes
90	N^6-[N-(N-acryloyl-1-methoxycarbonyl-5-aminopentyl)propioamide]	*19*	Yes
91	N^6-(6-aminohexyl)	*113*	Yes
92	N^6-(carboxymethyl)	*14*	Yes
93	N^6-[N-(6-aminohexyl)acetamide]	*14*	Yes
94	8-Br	*15–17*	Yes
95	8-(6-aminohexyl)amino	*15,16*	Yes
96	8-SH	*91*	Yes
97	1,N^6-etheno	*18*	Yes
98	N^6-(2,2,6,6-tetramethylpiperidin-4-yl-oxyl)	*114*	Yes
99	N^1-aminoethyl	*115*	Yes
100	N^6-aminoethyl	*115*	Yes
101	N^1-CH_2—CH(OH)—CH_2OH	*115*	Yes
102	N^6-CH_3—CH(OH)—CH_2OH	*115*	Yes

Reference	Enzymatically active
88,89	No
88,89	No
90	No
88,89	No
40,91	No
40,65,91	No
40	No
41,92	No
93–95	Yes
93–95	No
96	No
91	No
91	No
91	No
91	No
97	—
98	No
99	No
100	No
101	No
101	No
101	No

(*continued*)

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent	Reference	Enzymatically active
103	N^1-CH_2—$CH(OH)$—CH_2Cl	*115*	Yes
104	N^6-CH_2—$CH(OH)$—CH_2Cl	*115*	Yes
105	N^1-CH_2—$CH(OH)$—CH(O)CH_2	*115*	Yes
106	N^6-CH_2—$CH(OH)$—CH(O)CH_2	*115*	Yes
107	N^1-CH_2—$CH(OH)$—CH_2—O—$(CH_2)_4$—O—CH_2—CH(O)CH_2	*115*	Yes
108	N^6-CH_2—$CH(OH)$—CH_2—O—$(CH_2)_4$—O—CH_2—CH(O)CH_2	*115*	Yes
109	N^1-$CH_2CH(OH)$—$(CH_2)_4$—CH(O)CH—CH_2—O—CH_2—CH=CH_2	*115*	Yes
110	N^6-$CH_2CH(OH)$—$(CH_2)_4$—CH(O)CH—CH_2—O—CH_2—CH=CH_2	*115*	Yes
111	8-Azido	*116*	Yes
112	N^6-2-hydroxy-3-carboxypropyl	*117,118*	Yes
113	N^6-2-hydroxybutyrylpolyethyleneimine	*117,118*	Yes
114	8—NH—CH_3	*119*	Yes
115	8—NH—C_2H_5	*119*	Yes
116	8—$N(CH_3)_2$	*119*	Yes
B.	Replacement of the adenine ring		
117	Hypoxanthine	*1,2*	Yes
118	6-Mercaptopurine	*30,61*	Yes
119	Uracil	*120-122*	Yes
120	Cytosine	*122,123*	Yes
121	Nicotinamide	*5,120,122-124*	Yes
122	1,4-Dihydronicotinamide	*124*	Yes
123	Purine	*5,122,125*	Yes
124	Benzimidazole	*125-127*	Yes
125	2-Chloro-6-methylpurine	*125*	Yes
126	6-Methylpurine	*125*	Yes
127	3-Deazapurine	*127*	Yes
128	1-Deazapurine	*126,127*	Yes

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent
129	6-Methylmercaptopurine
130	Benzene
131	5-Iodouracil
132	5-Methylmercurythioinosine
133	4-Methyl-5-acetylimidazole
134	4-Methyl-5-bromacetylimidazol
135	Formycin
136	Tubercidin
137	3,N^4-Ethenocytosine
138	Thymine
139	2-Aminopurine
140	7-Deazapurine
141	Guanine
C.	Replacement of the ribose moiety
142	2-Deoxyribose
143	Methanol
144	Ethanol
145	Propanol
146	Butanol
147	L-Ribose
148	Arylazido-β-alanylribose
149	3-Deoxyribose
III.	Alteration in the pyrophosphate group
150	Monomethyl ester
151	Dimethyl ester
152	Triphosphate
IV.	Multiple alterations
153	3-Acetylpyridine-hypoxanthin
154	Pyridine-3-aldehyde-hypoxant dinucleotide
155	3-Carboxamido-4-(1-imidazol hypoxanthine dinucleotide
158	3-Acetylpyridine riboside dipl
157	Nicotinamide-thymidylate din
158	Nicotinamide riboside diphosj 6-aminohexyladenine
159	Adenosine-5′-diphosphoryl-4 tetramethylpiperidine-1-oxy

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent	Reference	Enzymatically active
129	6-Methylmercaptopurine	*30*	Yes
130	Benzene	*127–129*	Yes
131	5-Iodouracil	*121*	Yes
132	5-Methylmercurythioinosine	*31*	Yes
133	4-Methyl-5-acetylimidazole	*130*	Yes
134	4-Methyl-5-bromacetylimidazole	*32,131,132*	Yes
135	Formycin	*62,63*	Yes
136	Tubercidin	*63*	Yes
137	3,N^4-Ethenocytosine	*133*	Yes
138	Thymine	*122*	Yes
139	2-Aminopurine	*62*	Yes
140	7-Deazapurine	*62*	Yes
141	Guanine	*61,134*	Yes
	C. Replacement of the ribose moiety		
142	2-Deoxyribose	*120,135*	Yes
143	Methanol	*136*	Yes
144	Ethanol	*136*	Yes
145	Propanol	*136*	Yes
146	Butanol	*136*	Yes
147	L-Ribose	*137*	Yes
148	Arylazido-β-alanylribose	*33,138*	Yes
149	3-Deoxyribose	*63*	Yes
III.	Alteration in the pyrophosphate group		
150	Monomethyl ester	*139*	No
151	Dimethyl ester	*139*	No
152	Triphosphate	*120*	—
IV.	Multiple alterations		
153	3-Acetylpyridine-hypoxanthine	*140,141*	Yes
154	Pyridine-3-aldehyde-hypoxanthine-dinucleotide	*140,141*	Yes
155	3-Carboxamido-4-(1-imidazolyl)pyridine-hypoxanthine dinucleotide	*87*	No
158	3-Acetylpyridine riboside diphosphoribose	*123*	Yes
157	Nicotinamide-thymidylate dinucleotide	*120*	—
158	Nicotinamide riboside diphosphate-9-butyl-6-aminohexyladenine	*142*	Yes
159	Adenosine-5'-diphosphoryl-4-(2,2,6,6-tetramethylpiperidine-1-oxyl)	*143*	No

(*continued*)

TABLE I (*Continued*)

Analogs of NAD

Compound number	Substituent	Reference	Enzymatically active
160	6-Methylthiopurine-CH_2—(CONH—CH_2)—CH_2-nicotinamide	*144*	No
161	6-Methylthiopurine-CH_2—(CONH—CH_2)$_2$—CH_2-nicotinamide	*144*	No
162	6-Methylthiopurine-CH_2—CH_2—(CONH—CH_2)—CH_2-nicotinamide	*144*	No
163	4(3-Bromoacetylpyridinio)butyldiphospho-adenosine	*145*	Yes
164	4(3-Acetylpyridinio)butyldiphospho-adenosine	*145,146*	Yes
165	3(3-Bromoacetylpyridinio)propyl-diphosphoadenosine	*147*	No
166	3(4-Bromoacetylpyridinio)propyl-diphosphoadenosine	*148*	No
167	3(4-Acetylpyridinio)propyldiphospho-adenosine	*148*	No
168	3(3-Acetylpyridinio)propyldiphospho-adenosine	*145,146*	No
169	3(2-Acetylpyridinio)propyldiphospho-adenosine	*149*	No
170	3(2-Bromoacetylpyridinio)propyl-diphosphoadenosine	*149*	No
171	2-(3-Acetylpyridinio)ethyldiphospho-adenosine	*146*	No
172	5-(3-Acetylpyridinio)pentyldiphospho-adenosine	*146*	Yes
173	6-(3-Acetylpyridinio)hexyldiphospho-adenosine	*146*	No
174	Nicotinamide riboside-9-ethyl-6-trifluor-acetyl-aminohexyladenine	*142*	Yes
175	Nicotinamide riboside-5'-diphosphoryl-riboflavin	*150*	No
176	Nicotinamide riboside-5'-diphosphoryl-thiamin	*150*	No
177	Thionicotinamide xanthine dinucleotide	*151*	Yes
V.	Miscellaneous Derivatives		
178	α-Nicotinamide-β-adenine dinucleotide	*152*	No
179	β-Nicotinamide-α-adenine dinucleotide	*153*	Yes
180	Nicotinamide mononucleotide-3-iso-AMP	*154*	Yes
181	1,N^6-ethenoadenosine-5'-diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl)	*155*	No

Many of the pyridine nucleotide analc function as coenzymes in dehydrogenas tions. The notation "enzymatically activ question has been demonstrated to functi tion. Any derivative experimentally obse a dehydrogenase reaction is listed as not e of these cases, an exhaustive investigati performed. A cursory inspection of Tabl ing of pyridine nucleotides as coenzym less affected by alterations in the purine the molecule. In fact, all 64 derivatives adenosine portion of the NAD molecul coenzymatically in at least one dehydro

IV. PROPERTIES OF COENZYME AN

A. Spectral Properties

The absorbance spectrum of NAD exh which reflects the combined absorba nicotinamide and adenine minus a smal tions in either of the heterocyclic base absorbance maxima. This is readily nicotinamide ring has been converted tc second absorbance maximum at 340 nn oxidized dinucleotide derivatives when nicotinamide or adenine differ spectrally chosen examples are listed in Table II. ing from substitution in the adenin

TABLE II

Spectral Properties of Selected Pyridine Nucle

NAD analog
Nicotinamide 2-aminopurine dinucleotide
Nicotinamide 8-azidoadenine dinucleotide
Nicotinamide 1-deazapurine dinucleotide
3-Aminopyridine adenine dinucleotide
3-Diazoacetylpyridine adenine dinucleotide
5-Aminonicotinamide adenine dinucleotide

Many of the pyridine nucleotide analogs listed in Table I were observed to function as coenzymes in dehydrogenase-catalyzed oxidation–reduction reactions. The notation "enzymatically active" simply indicates that the analog in question has been demonstrated to function in at least one dehydrogenase reaction. Any derivative experimentally observed not to be enzymatically reduced in a dehydrogenase reaction is listed as not enzymatically active. However, in most of these cases, an exhaustive investigation of dehydrogenase reactions was not performed. A cursory inspection of Table I will reveal the fact that the functioning of pyridine nucleotides as coenzymes in dehydrogenase reactions is much less affected by alterations in the purine moiety than in the pyridinium region of the molecule. In fact, all 64 derivatives listed as containing alterations in the adenosine portion of the NAD molecule have been demonstrated to function coenzymatically in at least one dehydrogenase-catalyzed reaction.

IV. PROPERTIES OF COENZYME ANALOGS

A. Spectral Properties

The absorbance spectrum of NAD exhibits an absorbance maximum at 259 nm which reflects the combined absorbances of the two heterocyclic bases, nicotinamide and adenine minus a small hypochromic effect. Structural alterations in either of the heterocyclic bases can result in the appearance of new absorbance maxima. This is readily exemplified by NADH in which the nicotinamide ring has been converted to 1,4-dihydronicotinamide resulting in a second absorbance maximum at 340 nm. Spectral changes are also observed in oxidized dinucleotide derivatives when the chromophore substituted for either nicotinamide or adenine differ spectrally from the natural bases. A few randomly chosen examples are listed in Table II. The second absorbance maximum resulting from substitution in the adenine or nicotinamide portions of these

TABLE II

Spectral Properties of Selected Pyridine Nucleotide Analogs

NAD analog	Absorption maxima (nm)	Reference
Nicotinamide 2-aminopurine dinucleotide	265, 305	*62*
Nicotinamide 8-azidoadenine dinucleotide	264, 274	*116*
Nicotinamide 1-deazapurine dinucleotide	242, 275	*126*
3-Aminopyridine adenine dinucleotide	257, 331	*20*
3-Diazoacetylpyridine adenine dinucleotide	258.5, 307	*25*
5-Aminonicotinamide adenine dinucleotide	260, 348	*40*

	Reference	Enzymatically active
	144	No
	144	No
	144	No
	145	Yes
	145,146	Yes
	147	No
	148	No
	148	No
	145,146	No
	149	No
	149	No
	146	No
	146	Yes
	146	No
	142	Yes
	150	No
	150	No
	151	Yes
	152	No
	153	Yes
	154	Yes
	155	No

dinucleotides can be used as a means of identifying these derivatives. In the case of 3-aminopyridine adenine dinucleotide, the free base, 3-aminopyridine, does not absorb maximally at 331 nm and therefore, the appearance of absorbance at this wavelength with time can be used to monitor the rate of formation of this analog. Since 3-aminopyridine is an effective substrate in the NAD-transglycosidation reaction catalyzed by several different NADases, the spectral difference between the free base and the pyridinium form in the analog provides a direct spectrophotometric method for studying transglycosidation reactions with purified NADases.

A number of the NAD analogs listed in Table I contain nicotinamide or a pyridine base having an oxidation-reduction potential similar to that of nicotinamide in the pyridinium form. Differences in the spectral properties of the reduced forms of these analogs occur mainly in those compounds containing pyridine bases other than nicotinamide. Those analogs containing nicotinamide and altered only in the purine moiety, upon reduction, exhibit a second absorbance maximum very close to the 340 nm maximum observed with NADH. The reduction of analogs containing pyridine bases other than nicotinamide can result in new absorbance maxima quite different from NADH. In this respect the second absorbance maxima for the reduced analogs containing 3-acetylpyridine, thionicotinamide, 3-pyridylacrylamide, and 3-chloroacetylpyridine occur at 365 nm, 398 nm, 385 nm, and 374.5 nm, respectively [*23,25,27*]. These derivatives are cited merely as examples of reduced analogs having spectral properties differing from NADH. A number of other pyridine-substituted analogs listed in Table I have also been demonstrated to differ spectrally from NADH in their reduced forms. These derivatives have important applications to studies of dehydrogenases. They provide alternate means of assaying dehydrogenases in the presence of substances absorbing sufficiently at 340 nm to interfere with the monitoring of NADH formation. For example, in order to evaluate the coenzyme-competitive inhibition of chicken liver 3-phosphoglycerate dehydrogenase by 3-aminopyridine adenine dinucleotide which absorbs at 331 nm, initial velocities of the reduction of 3-acetylpyridine adenine dinucleotide were measured at 365 nm avoiding the spectral interference by the inhibitor at 340 mm [*179*]. In this respect, 3-acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide have been substituted for the native coenzyme in a number of studies in order to provide a more accurate measurement of initial velocities in dehydrogenase-catalyzed reactions.

Several NAD analogs have been prepared which contain fluorescing heterocyclic bases in either the pyridine or purine portion of the dinucleotide molecule. A few of the more frequently cited fluorescing analogs are listed in Table III. The fluorescence of the fluorophores of these oxidized dinucleotides has been observed to be significantly quenched through intramolecular ring-ring interactions. Spectroscopic studies of several of these fluorescing analogs indi-

TABLE III

Fluorescent Properties of Selected NAD Analogs

Analog
Nicotinamide 2-aminopurine dinucleotide
Nicotinamide 7-deazapurine dinucleotide
Nicotinamide formycin dinucleotide
Nicotinamide 1,N^6-ethenoadenine dinucleotide
5-Aminonicotinamide adenine dinucleotide
3-Aminopyridine adenine dinucleotide

cate the quenching of fluorescence to b
folded conformations of these
[*20,40,62,133,170,180-184*]. Processes
these dinucleotides result in significant
from 3- to 13-fold in these derivatives.
5-aminonicotinamide and 3-aminopyridi
tic repulsion of positively charged hetero
cence enhancement that can be used t
process. Hydrolysis of the fluorescing a
NADases or pyrophosphatases likewis
which has been helpful in developing f

B. Oxidation-Reduction Properties

NAD analogs substituted only in the
oxidation-reduction potentials very c
Oxidation-reduction potentials differin
in analogs altered in the NMN portion
altered in the pyridinium ribose moiety
from NAD in oxidation-reduction pot
measurement of cyanide adduct dissoci
effects are observed when the electroni
of the pyridine ring is changed. For
carbonyl analogs, 3-acetylpyridine ade
adenine dinucleotide, exhibit equilib
drogenase reactions 200 times greate
indicating an oxidation-reduction pote
mV for NAD [*186,187*]. An E_0' of -2
rium studies for the 3-benzoylpyridin

TABLE III

Fluorescent Properties of Selected NAD Analogs

Analog	Excitation wavelength (nm)	Emission wavelength (nm)	Reference
Nicotinamide 2-aminopurine dinucleotide	310	370	*62*
Nicotinamide 7-deazapurine dinucleotide	290	400	*62*
Nicotinamide formycin dinucleotide	295	340	*62*
Nicotinamide $1,N^6$-ethenoadenine dinucleotide	305	410	*18*
5-Aminonicotinamide adenine dinucleotide	348	445	*40*
3-Aminopyridine adenine dinucleotide	331	420	*20*

cate the quenching of fluorescence to be related to the presence of stacked or folded conformations of these dinucleotides in aqueous solution [*20,40,62,133,170,180–184*]. Processes that disrupt the folded conformations of these dinucleotides result in significant enhancement of fluorescence varying from 3- to 13-fold in these derivatives. Protonation of the adenine ring of the 5-aminonicotinamide and 3-aminopyridine analogs [*20,40*] results in electrostatic repulsion of positively charged heterocyclic bases with a concomitant fluorescence enhancement that can be used to evaluate the pK'_a of this protonation process. Hydrolysis of the fluorescing analogs listed in Table III as catalyzed by NADases or pyrophosphatases likewise results in fluorescence enhancement which has been helpful in developing fluorimetric assays for these enzymes.

B. Oxidation–Reduction Properties

NAD analogs substituted only in the adenine portion of the molecule exhibit oxidation–reduction potentials very close to that observed for NAD itself. Oxidation–reduction potentials differing from that of NAD have been observed in analogs altered in the NMN portion of the molecule. A number of analogs altered in the pyridinium ribose moiety have been demonstrated to differ slightly from NAD in oxidation–reduction potential [*4,109,111,145*] as estimated by measurement of cyanide adduct dissociation constants [*185*]. As expected, larger effects are observed when the electronic character of the group in the 3-position of the pyridine ring is changed. For example, the more electron-withdrawing carbonyl analogs, 3-acetylpyridine adenine dinucleotide and pyridine-3-aldehyde adenine dinucleotide, exhibit equilibrium constants for reduction in dehydrogenase reactions 200 times greater than that observed with NAD [*27,28*] indicating an oxidation–reduction potential of −248 mV as compared to −320 mV for NAD [*186,187*]. An E'_0 of −248 mV was also obtained through equilibrium studies for the 3-benzoylpyridine and 3-isobutyrylpyridine analogs [*64*].

The less electron-withdrawing thionicotinamide analog exhibited an E_0' of −285 mV while the greater electron-withdrawing 3-cyanopyridine analog was even more favorably reduced than the carbonyl derivatives [24]. In contrast, NAD analogs containing pyridine, β-picoline or 3-methylpyridyl carbinol not having electron-withdrawing substituents are not reduced enzymatically or chemically with dithionite and do not react to form addition products in the 4-position of the pyridine ring [27]. The vinyl analog of NAD, 3-pyridylacrylamide adenine dinucleotide, was reduced chemically with dithionite but did not serve as a coenzyme for a number of dehydrogenases tested [64]. This analog did serve as an acceptor molecule in the pig heart NADH diaphorase reaction from which an E_0' of −347 mV was determined. Equilibrium studies of diaphorase reactions can provide a means of determining oxidation-reduction potentials for analogs not functional in dehydrogenase-catalyzed reactions.

V. APPLICATIONS

A. Enzymatic Reactions

In considering the functioning of NAD analogs with dehydrogenases and other pyridine nucleotide-requiring enzymes, it is advantageous to distinguish between analog-enzyme interactions that result in successful catalytic processes and those interactions that although selective, lead solely to competitive inhibition. In both cases information can be gained concerning selective interactions between enzyme functional groups and the dinucleotide molecule. A number of excellent reviews [4,188,189] have discussed the functioning of coenzyme analogs with dehydrogenases. Early studies concentrated upon the question of which groups of the NAD molecule were essential for the functioning of NAD as a coenzyme in dehydrogenase-catalyzed reactions. As noted above and indicated by the listing in Table I, the AMP portion of NAD can be drastically altered without the total loss of coenzyme function in dehydrogenase reactions. The functioning of a large number of coenzyme analogs modified in the purine portion of the molecule as measured in reactions catalyzed by horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase, and pig heart lactate dehydrogenase has been described by Woenckhaus [4]. The substitution of the adenine by a variety of aromatic derivatives gives rise to variations in both K_m and V_{max} values; however, in general, appreciable coenzyme activity was observed in each case. These variations in kinetic parameters document an importance for the adenine ring in the functioning of NAD in dehydrogenase reactions; however, if one completely removes the adenine ring as in nicotinamide riboside-5'-pyrophosphate-5''-ribose [123], the molecule still functions with the three enzymes mentioned above. The effectiveness of this derivative as a coenzyme is drastically reduced, and consid-

erably lower V_{max} values and higher K_m
wide variety of aromatic residues that car
tion of coenzyme activity is consistent wi
in dehydrogenases, observed through X-r
with the adenine moiety of NAD in stabil
can vary in size and composition from on
effects resulting from modification of d
likewise vary. Although the AMP portion
nonfunctional part of the coenzyme, the
enzyme can be required for full activity
tion of the adenine ring itself, modificati
fere with the proper orientation of the N
have reported such effects in the functio
Modifications of the functional NMN
expected, have greater effects on the coe
the 81 derivatives listed in Table I contai
moiety, only 16 have been demonstra
catalyzed reactions. Since most of the c
carbonyl function in the 3-position of th
early studies that a 3-position carbon do
gen was an absolute requirement for func
coenzymatic functioning of the 3-cyanop
major exception to this concept; ho
3-halopyridine analogs of NAD are redu
tion of other factors. Presumably, 3-po
power sufficient to activate the 4-posi
coenzymatically active analogs. In st
3-iodopyridine analogs. Abdallah *et a*
forms of these analogs do not exhibit the
region. The appearance of such maxim
the functioning of NAD analogs in dehy
erly demonstrated the functioning of th
liver alcohol dehydrogenase by using ci
ing the formation of cinnamaldehyde wh
[190,191]. This method provides a sen
ing of NAD analogs which upon redu
characteristic spectral changes.

The fact that pyridine nucleotide
electron-withdrawing groups in the 3-
more negative oxidation-reduction pot
advantage in the study of dehydrogena
dehydrogenation reactions involving

amide analog exhibited an E_0' of −285
ng 3-cyanopyridine analog was even
l derivatives [*24*]. In contrast, NAD
r 3-methylpyridyl carbinol not having
reduced enzymatically or chemically
dition products in the 4-position of the
NAD, 3-pyridylacrylamide adenine
ith dithionite but did not serve as a
s tested [*64*]. This analog did serve as
DH diaphorase reaction from which an
um studies of diaphorase reactions can
n-reduction potentials for analogs not
actions.

nalogs with dehydrogenases and other
s advantageous to distinguish between
uccessful catalytic processes and those
olely to competitive inhibition. In both
ng selective interactions between en-
ide molecule. A number of excellent
unctioning of coenzyme analogs with
l upon the question of which groups of
unctioning of NAD as a coenzyme in
ted above and indicated by the listing
be drastically altered without the total
e reactions. The functioning of a large
the purine portion of the molecule as
r liver alcohol dehydrogenase, yeast
ite dehydrogenase has been described
the adenine by a variety of aromatic
h K_m and V_{max} values; however, in
as observed in each case. These var-
importance for the adenine ring in the
actions; however, if one completely
e riboside-5′-pyrophosphate-5″-ribose
three enzymes mentioned above. The
me is drastically reduced, and consid-

erably lower V_{max} values and higher K_m values are observed in each case. The wide variety of aromatic residues that can be substituted for adenine with retention of coenzyme activity is consistent with the presence of hydrophobic regions in dehydrogenases, observed through X-ray crystallographic studies, that interact with the adenine moiety of NAD in stabilizing binary complexes. These regions can vary in size and composition from one dehydrogenase to another and specific effects resulting from modification of different parts of the adenine ring can likewise vary. Although the AMP portion of NAD is frequently referred to as the nonfunctional part of the coenzyme, the proper orientation of this moiety on the enzyme can be required for full activity [*62,121,154*]. In addition to modification of the adenine ring itself, modification of the adenine ribose can also interfere with the proper orientation of the NAD molecule. Suhadolnik *et al.* [*63*] have reported such effects in the functioning of 2′- and 3′-deoxy-NAD.

Modifications of the functional NMN portion of the coenzyme molecule, as expected, have greater effects on the coenzyme functioning of the molecule. Of the 81 derivatives listed in Table I containing alterations solely in the pyridinium moiety, only 16 have been demonstrated to be reduced in dehydrogenase-catalyzed reactions. Since most of the coenzymatically active analogs contain a carbonyl function in the 3-position of the pyridine ring, it was speculated from early studies that a 3-position carbon double bonded to oxygen, sulfur, or nitrogen was an absolute requirement for functioning in dehydrogenase reactions. The coenzymatic functioning of the 3-cyanopyridine analog [*26*] does not represent a major exception to this concept; however, recent observations [*22*] that 3-halopyridine analogs of NAD are reduced enzymatically do require consideration of other factors. Presumably, 3-position groups with electron-withdrawing power sufficient to activate the 4-position of the pyridine ring can result in coenzymatically active analogs. In studies of the 3-chloro-, 3-bromo-, and 3-iodopyridine analogs, Abdallah *et al.* [*22*] demonstrated that the reduced forms of these analogs do not exhibit the usual absorbance maxima in the 300 nm region. The appearance of such maxima has been used routinely to demonstrate the functioning of NAD analogs in dehydrogenase reactions. These authors cleverly demonstrated the functioning of the 3-halopyridine derivatives with horse liver alcohol dehydrogenase by using cinnamyl alcohol as substrate and monitoring the formation of cinnamaldehyde which exhibits a high absorbance at 290 nm [*190,191*]. This method provides a sensitive tool for investigating the functioning of NAD analogs which upon reduction would not be expected to exhibit characteristic spectral changes.

The fact that pyridine nucleotide analogs substituted with more highly electron-withdrawing groups in the 3-position of the pyridinium ring exhibit a more negative oxidation-reduction potential than that of NAD, can be a distinct advantage in the study of dehydrogenase-catalyzed reactions. The equilibria of dehydrogenation reactions involving such analogs are shifted in favor of the

formation of oxidized substrate, thus producing at any given pH increased amounts of the reduced pyridine nucleotide which is normally used to monitor the reaction spectrophotometrically. In a recent study of α secondary isotope effects in yeast alcohol dehydrogenase reactions, Welsh *et al.* [*192*] used 3-acetylpyridine adenine dinucleotide as the coenzyme to provide a greater percent conversion of alcohol to aldehyde at equilibrium. Previous studies [*193,194*] had demonstrated that the use of this analog favored aldehyde production by two orders of magnitude.

Some of the applications of NAD analogs based on differing spectral properties have been noted. Levy and Daouk [*195*] recently reported a new application for the thionicotinamide analogs of NAD and NADP. These authors described a method for the simultaneous assay of NAD- and NADP-linked reactions of dehydrogenases which can utilize both coenzymes. *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase exhibits a dual coenzyme specificity reacting with either NAD or NADP. The two distinct coenzyme reactions were reported to be catalyzed by two different isomers of the enzyme existing in equilibrium [*196*]. Levy and Daouk [*195*] demonstrated that assay mixtures containing both NAD and thionicotinamide adenine dinucleotide phosphate (or thionicotinamide adenine dinucleotide and NADP) could be used to simultaneously measure both coenzyme reactions by monitoring the reduction of the dinucleotides at both 340 and 400 nm. Using this method, these authors demonstrated that increasing the concentration of substrate, glucose 6-phosphate, promoted the NAD-linked reaction relative to the NADP-linked reaction. This phenomenon was not observed in the conventional single coenzyme assays of the enzyme. The authors discuss these observations in relation to *in vivo* regulation of this enzyme with respect to the catabolic and anabolic roles of NAD and NADP, respectively, in this organism. They further suggest that the dual wavelength assay should be applicable to the study of many other dehydrogenases exhibiting dual coenzyme specificity.

NAD analogs containing a second substituent on the nicotinamide ring have been observed to be inactive as coenzymes in dehydrogenase reactions. Questions have been raised as to whether or not the inactivity of these derivatives should be attributed to steric effects or changes in oxidation-reduction potential. In studies of the 5-methylnicotinamide and 5-aminonicotinamide analogs of NAD, Walter and Kaplan [*40*] concluded that steric effects predominated in the inactivity of these analogs as coenzymes. More recently, Trommer *et al.* [*96*] prepared 5-carboxynicotinamide adenine dinucleotide and demonstrated the oxidation-reduction potential of this analog to be −262 mV. Being more electropositive than NAD, the analog could be expected to be reduced in dehydrogenase reactions. When tested with three different dehydrogenases no reduction of the 5-carboxynicotinamide analog was observed. Since coenzyme-competitive inhibition was observed with this compound, the inactivity as a coenzyme was attributed predominantly to steric interactions.

Modification of the nicotinamide rib
affect both the oxidation-reduction po
proper orientation of the coenzyme in b
stitution of this ribose by glucose results
dehydrogenases [*111*]. Aliphatic group
stituted for ribose in the coenzymat
dinucleotide [*145,146*]. The importan
pyridine ring and the pyrophosphate mo
and pentyl derivatives functioned in d
propyl, and hexyl derivatives did not [

There are a large number of studies i
investigate binding processes of dehydr
requiring enzymes. Binding properties l
inhibitor analysis and more directly thro
labeling, fluorescence titration, and X-r
exemplifies the use of NAD analogs to i
octopine dehydrogenase by Olomucki
interaction of this enzyme with 21 N
molecule, AMP, ADP, and ADP-ribos
mine which part of the coenzyme mole
on octopine dehydrogenase and to eluc
site of this enzyme. It was observed t
positions of the adenine ring did not int
and the results obtained were consiste
which indicated nonpolar interactions
adenine moiety. A comparison of the
indicated that interactions with the py
ribose were also important in the over
the binding of analogs altered in the py
hydrogen bonding between the group
side-chain groups. Larger substituents
as thioamide, propionyl, and chloracet
The presence of the methyl gro
5-methylnicotinamide analogs did not
create sufficient steric hindrance to pre
This application of coenzyme analogs
cerning the orientation and interactions
site of this enzyme.

Fluorescing coenzyme analogs add
zyme binding to dehydrogenases. T
fluorescing derivatives in place of ni
strated to be partially quenched throug
conformations of these molecules. A

roducing at any given pH increased
de which is normally used to monitor
recent study of α secondary isotope
reactions, Welsh *et al.* [*192*] used
he coenzyme to provide a greater per-
le at equilibrium. Previous studies
f this analog favored aldehyde produc-

ogs based on differing spectral prop-
195] recently reported a new applica-
NAD and NADP. These authors de-
say of NAD- and NADP-linked reac-
both coenzymes. *Leuconostoc mesen-*
ase exhibits a dual coenzyme speci-
. The two distinct coenzyme reactions
rent isomers of the enzyme existing in
'5] demonstrated that assay mixtures
le adenine dinucleotide phosphate (or
l NADP) could be used to simultane-
by monitoring the reduction of the
ng this method, these authors demon-
f substrate, glucose 6-phosphate, pro-
the NADP-linked reaction. This phe-
tional single coenzyme assays of the
ations in relation to *in vivo* regulation
olic and anabolic roles of NAD and
They further suggest that the dual
to the study of many other dehy-
ificity.

tituent on the nicotinamide ring have
:s in dehydrogenase reactions. Ques-
ıot the inactivity of these derivatives
nges in oxidation-reduction potential.
ınd 5-aminonicotinamide analogs of
that steric effects predominated in the
More recently, Trommer *et al.* [*96*]
dinucleotide and demonstrated the
g to be −262 mV. Being more elec-
be expected to be reduced in dehy-
e different dehydrogenases no reduc-
'g was observed. Since coenzyme-
this compound, the inactivity as a
steric interactions.

Modification of the nicotinamide ribose of NAD has been demonstrated to affect both the oxidation-reduction potential of the pyridinium ring and the proper orientation of the coenzyme in binding to dehydrogenases [*4*]. The substitution of this ribose by glucose results in an analog that does not function with dehydrogenases [*111*]. Aliphatic groups of varying chain length have been substituted for ribose in the coenzymatically active 3-acetylpyridine adenine dinucleotide [*145,146*]. The importance of the distance between the acetylpyridine ring and the pyrophosphate moiety is indicated by the fact that the butyl and pentyl derivatives functioned in dehydrogenase reactions while the ethyl, propyl, and hexyl derivatives did not [*145,146,197*].

There are a large number of studies in which NAD analogs have been used to investigate binding processes of dehydrogenases and other pyridine nucleotide-requiring enzymes. Binding properties have been determined kinetically through inhibitor analysis and more directly through a number of techniques such as spin labeling, fluorescence titration, and X-ray crystallography. One such study that exemplifies the use of NAD analogs to investigate binding processes is a study of octopine dehydrogenase by Olomucki *et al.* [*198*]. These authors studied the interaction of this enzyme with 21 NAD analogs plus fragments of the NAD molecule, AMP, ADP, and ADP-ribose. These studies were designed to determine which part of the coenzyme molecule was essential for correct positioning on octopine dehydrogenase and to elucidate properties of the coenzyme binding site of this enzyme. It was observed that substitution at the N-1, N-6, or N-8 positions of the adenine ring did not interfere with normal binding to the enzyme and the results obtained were consistent with earlier fluorimetric studies [*199*] which indicated nonpolar interactions to be important in the binding of the adenine moiety. A comparison of the binding of AMP, ADP, and ADP-ribose indicated that interactions with the pyrophosphate group and the nicotinamide ribose were also important in the overall coenzyme binding process. Studies of the binding of analogs altered in the pyridinium moiety demonstrated a need for hydrogen bonding between the group in the 3-position of the ring and protein side-chain groups. Larger substituents in the 3-position of the pyridine ring such as thioamide, propionyl, and chloracetyl decreased the effectiveness of binding. The presence of the methyl group in the 4-methylnicotinamide and 5-methylnicotinamide analogs did not prevent binary complex formation but did create sufficient steric hindrance to prevent the formation of ternary complexes. This application of coenzyme analogs provided considerable information concerning the orientation and interactions of coenzyme and substrate at the catalytic site of this enzyme.

Fluorescing coenzyme analogs add another dimension to the studies of coenzyme binding to dehydrogenases. The fluorescence of analogs containing fluorescing derivatives in place of nicotinamide or adenine has been demonstrated to be partially quenched through intramolecular interactions in the folded conformations of these molecules. A number of studies [*169,179,181,182,*

200-205] can be cited to indicate that the fluorescing analogs, nicotinamide-1, N^6-ethenoadenine dinucleotide (ϵ-NAD), 3-aminopyridine adenine dinucleotide and the native coenzyme exist to a degree in a folded conformation in aqueous solutions. Any process that results in a shift in the conformational equilibrium would in the case of the fluorescing analogs be accompanied by an enhancement of fluorescence. In studies of the interactions of ϵ-NAD with dehydrogenases [*179,183,199,205-209*] fluorescence enhancement on binding has been observed. Luisi *et al.* [*183*] have reported a 10- to 13-fold enhancement of fluorescence on the binding of ϵ-NAD to several different dehydrogenases. These authors interpreted the fluorescence enhancement to indicate the binding of ϵ-NAD in an open conformation on all of the enzymes studied. This argument was based on the fact that the degree of fluorescence enhancement was essentially the same with each enzyme studied and essentially the same as that observed on hydrolysis of the ϵ-NAD molecule catalyzed by snake venom phosphodiesterase. An explanation based on environmental effects related to the binding of the ethenoadenine moiety in a hydrophobic region was considered less likely due to the large degree of fluorescence enhancement and the fact that the fluorescence of 5′-ϵ-AMP was observed to be relatively insensitive to changes in hydrophobicity. Thus, the fluorescence of both free and bound coenzyme analogs can be used to investigate the interactions that take place at coenzyme binding sites. Gafni [*210*] has reported the use of iodide and other small molecules to study the quenching of fluorescence of ϵ-NAD bound to dehydrogenases as a means of investigating the accessibility of coenzyme binding sites to the external solvent. The literature concerning spectral changes on the binding of coenzymes and coenzyme analogs to enzymes is quite extensive. One of the many studies available that might be cited as related to the above discussion of conformation of bound coenzyme is that reported by Woenckhaus and Scherr [*121*]. These authors demonstrated through spectral studies that the reduced nicotinamide iodouracil dinucleotide was bound to lactate and alcohol dehydrogenases in an unfolded conformation.

Coenzyme analogs have been very useful in studies of pyridine nucleotide transhydrogenases. Since the extinction coefficients at 340 nm for NADPH and NADH are essentially the same, the reversible reaction of NADPH with NAD to form NADP and NADH would not result in a significant change in absorption at 340 nm. Stein *et al.* [*211*] reported the application of the 3-acetylpyridine analogs of NAD and NADP for spectrophotometric studies of these reactions. Since reduced 3-acetylpyridine adenine dinucleotide absorbs maximally at 365 nm with a slightly higher extinction coefficient than that of NADH at 340 nm [*211,212*], the reduction of 3-acetylpyridine adenine dinucleotide by NADPH can be readily followed spectrophotometrically. This reduction is usually followed at 375 nm where no contribution from NADPH occurs. This method has been used to study a variety of transhydrogenases and can be exemplified by studies of the transhydrogenase activity of submitochondrial particles [*213,214*].

The demonstration of a 400 nm absorba
the thionicotinamide analogs of NAD an
these derivatives also for the assay of
Kaplan [*215*] have demonstrated the app
transhydrogenase-catalyzed reactions a
number of transhydrogenase studies [*21*
of azotobacter transhydrogenase [*220*],
tive and the thionicotinamide analogs ha
thionicotinamide adenine dinucleotide p
transhydrogenase activity of yeast glutat

Pyridine nucleotide analogs have als
properties of other pyridine nucleotide-re
et al. [*50*] utilized a number of pyridine
the mechanism of action of solubiliz
Through such studies this enzyme was
possessing a carbonyl function at the
authors report no correlation between th
resulting pyridine base and discuss thes
limiting step of the catalyzed reaction.

The hydrolysis of pyridine nucleoti
glycohydrolases, nucleotide pyrophospt
conveniently monitored fluorimetrically
substrate. The bond-breaking processes
enhancement of fluorescence due to the
quenching interactions. Nicotinamide 1,
a substrate for bovine seminal fluid nucle
for this compound measured fluorimetri
titrimetrically [*222*]. 3-Aminopyridine a
substrate for this enzyme and either of th
to assay low concentrations of the enzyn
tive assay procedures, other advantages i
In studies comparing the properties of
erythrocytes to those in erythrocyte gh
assay proved unsuccessful due to the rel
[*56,223*]. Since nicotinamide 1,N^6-ethe
substrate for this enzyme, the compar
fluorescence techniques. The fact that t
the same as NAD in most systems mak
studies of other pyridine nucleotide re
recent study of human glucose-6-phospt
1,N^6-ethenoadenine dinucleotide phosph
cally the tight binding of phospho

: fluorescing analogs, nicotinamide-1,
3-aminopyridine adenine dinucleotide
e in a folded conformation in aqueous
hift in the conformational equilibrium
gs be accompanied by an enhancement
tions of ϵ-NAD with dehydrogenases
ncement on binding has been observed.
3-fold enhancement of fluorescence on
dehydrogenases. These authors inter-
licate the binding of ϵ-NAD in an open
1. This argument was based on the fact
ent was essentially the same with each
as that observed on hydrolysis of the
m phosphodiesterase. An explanation
e binding of the ethenoadenine moiety
less likely due to the large degree of
at the fluorescence of 5′-ϵ-AMP was
ges in hydrophobicity. Thus, the fluo-
analogs can be used to investigate the
inding sites. Gafni [210] has reported
to study the quenching of fluorescence
neans of investigating the accessibility
olvent. The literature concerning spec-
and coenzyme analogs to enzymes is
ailable that might be cited as related to
bound coenzyme is that reported by
thors demonstrated through spectral
racil dinucleotide was bound to lactate
d conformation.

ful in studies of pyridine nucleotide
efficients at 340 nm for NADPH and
ible reaction of NADPH with NAD to
n a significant change in absorption at
application of the 3-acetylpyridine
otometric studies of these reactions.
nucleotide absorbs maximally at 365
icient than that of NADH at 340 nm
ine adenine dinucleotide by NADPH
ically. This reduction is usually fol-
m NADPH occurs. This method has
ogenases and can be exemplified by
submitochondrial particles [213,214].

The demonstration of a 400 nm absorbance maximum for the reduced forms of the thionicotinamide analogs of NAD and NADP [68,69], suggested the use of these derivatives also for the assay of transhydrogenase activity. Cohen and Kaplan [215] have demonstrated the application of these analogs for the study of transhydrogenase-catalyzed reactions and this method has been used for a number of transhydrogenase studies [216-219]. In certain cases, as in the study of azotobacter transhydrogenase [220], the 3-acetylpyridine analogs were inactive and the thionicotinamide analogs had to be used. Moroff *et al.* [221] used thionicotinamide adenine dinucleotide phosphate to demonstrate and study the transhydrogenase activity of yeast glutathione reductase.

Pyridine nucleotide analogs have also been used successfully to study the properties of other pyridine nucleotide-requiring enzymes. For example, Schuber *et al.* [50] utilized a number of pyridine-substituted NAD analogs in a study of the mechanism of action of solubilized calf spleen NAD glycohydrolase. Through such studies this enzyme was demonstrated to be specific for analogs possessing a carbonyl function at the 3-position of the pyridine ring. These authors report no correlation between the rate of hydrolysis and the pK'_a of the resulting pyridine base and discuss these observations with respect to the rate-limiting step of the catalyzed reaction.

The hydrolysis of pyridine nucleotide coenzymes as catalyzed by NAD glycohydrolases, nucleotide pyrophosphatases, and phosphodiesterases can be conveniently monitored fluorimetrically by employing a fluorescing analog as substrate. The bond-breaking processes catalyzed by these enzymes result in an enhancement of fluorescence due to the release of intramolecular fluorescence quenching interactions. Nicotinamide $1,N^6$-ethenoadenine dinucleotide serves as a substrate for bovine seminal fluid nucleotide pyrophosphatase and the K_m value for this compound measured fluorimetrically did not differ from that measured titrimetrically [222]. 3-Aminopyridine adenine dinucleotide also functions as a substrate for this enzyme and either of these two fluorescing analogs can be used to assay low concentrations of the enzyme. In addition to providing more sensitive assay procedures, other advantages in using fluorescing analogs can be cited. In studies comparing the properties of NAD glycohydrolase activity in intact erythrocytes to those in erythrocyte ghosts, the normally employed titrimetric assay proved unsuccessful due to the release of metabolites from the intact cells [56,223]. Since nicotinamide $1,N^6$-ethenoadenine dinucleotide is an excellent substrate for this enzyme, the comparison of properties was achieved using fluorescence techniques. The fact that the ethenoadenine analog behaves much the same as NAD in most systems makes this compound a good candidate for studies of other pyridine nucleotide reactions such as ADP-ribosylation. In a recent study of human glucose-6-phosphate dehydrogenase [224], nicotinamide $1,N^6$-ethenoadenine dinucleotide phosphate was used to demonstrate fluorimetrically the tight binding of phospho-ADP-ribose to the enzyme. Since

3-aminopyridine adenine dinucleotide does not appear to function as a substrate with NAD glycohydrolases, an enzyme-catalyzed enhancement of fluorescence with this analog can serve to indicate bond breakage at other than the pyridine ribosidic linkage.

B. Site-Labeling Studies

Site labeling or affinity labeling has been used successfully for many years as an experimental tool for the covalent modification of functional groups of amino acid residues located in or nearby the active sites of enzymes. This experimental approach [225] involves the incorporation of chemically reactive groups into compounds structurally analogous to substrates or cofactors of a given enzyme. Selective binding of these compounds to enzyme sites directs the covalent modification process to those functional groups on the enzyme existing in or nearby the site involved. Enzyme functional groups identified in this fashion are thought to be those involved in the binding or catalytic processes of the enzyme. Site-labeling reagents have been developed which contain functional groups capable of a variety of reactions such as alkylation, azo coupling, Schiff-base formation and photochemical reactions.

A number of NAD and NADP analogs have been synthesized for the specific application of the site-labeling of dehydrogenases and other pyridine nucleotide-requiring enzymes. Due to the nature of the reactive group incorporated into these analogs, selective modification of specific enzyme functional groups has been achieved; however, in the case of photolabile reagents, possibilities for the modification of a greater variety of enzyme functional groups have been demonstrated. Both experimental approaches are important for the investigation of functional groups at enzyme sites. A number of photolabile pyridine nucleotide analogs designed to photogenerate reactive nitrene and carbene intermediates have been studied as site-labeling reagents for dehydrogenases [33,73,74,86,116, 138,226]. Questions have been raised concerning complications due to non-specific labeling by certain photolabile reagents [227,228] especially with respect to those not exhibiting sufficiently tight binding to receptor sites. In studies of the 3-diazirine analog of NAD (Table I, No.48), Standring and Knowles [86] discuss the relative effectiveness of carbene and nitrene generating reagents.

Most of the pyridine nucleotide analogs prepared as site-labeling reagents of dehydrogenases have been alkylating reagents and for the most part halomethyl ketone derivatives. The incorporation of such groups into pyridine nucleotides is a natural extension of the classic studies of Shaw and coworkers [229-231] on the development of *p*-toluenesulfonyllysyl chloromethyl ketone (TLCK) and *p*-toluenesulfonylphenylalanyl chloromethyl ketone (TPCK) as site-labeling reagents for trypsin and chymotrypsin, respectively. A number of pyridine nucleotide analogs containing an acetyl group in the pyridinium or purine portion of the molecule can be directly converted to bromoacetylated analogs by bromination [147]. 3-Chloroacetylpyridine aden
pyridine adenine dinucleotide phosphat
chlorination of the corresponding 3-diaz
acetyl dinucleotide derivatives serve as l
reagents with dehydrogenases. These d
cleophilic groups located at active sites
have been observed in their behavior
ample, the 3-bromoacetylpyridine analo
substituted by alkyl groups [145,147]
nucleotide do not inactivate lactate deh
ever, when the bromoacetyl group is i
pyridinio)propyladenosine pyrophosph
lactate dehydrogenase was observed [
reaction suggested selective modificati
enzyme.

Yeast alcohol dehydrogenase, due
sulfhydryl groups appears to be extrer
analogs of NAD. Nicotinamide 5-bro
[131]. 3-(4-bromoacetylpyridinio)-
adenosine diphosphate inactivate yeast
ification of cysteine-43 of this enzyme
plete inactivation of yeast alcohol deh
the incorporation of 4 moles of reage
site [234-236]. 4-(3-Bromoacetylpyr
and 3-chloroacetylpyridine adenine din
dehydrogenase. With the exception of
phosphate, these halomethyl ketone an
horse liver alcohol dehydrogenase in a
evidence exists indicating yeast and
homologous proteins sharing many pr
closely associated essential sulfhydryl
region, presumably serving as ligands
groups have been identified as cystein
[238] and cysteine-46 and cysteine-17
amide 4-methyl-5-bromoacetylimidaz
teine-43 of the yeast enzyme; howeve
drogenase by this reagent involves the
homologous cysteine-46 [131]. The
inactivation of these two dehydroge
adenosine diphosphate; however, ir
bromoacetylpyridinio)butyladenosine
of cysteine-43 of the yeast enzyme a
enzyme [244]. In addition, 3-(3-br

tion [147]. 3-Chloroacetylpyridine adenine dinucleotide [25] and 3-chloroacetylpyridine adenine dinucleotide phosphate [232] have been prepared through the chlorination of the corresponding 3-diazoacetylpyridine analogs. Most of the haloacetyl dinucleotide derivatives serve as both hydrogen acceptors and site-labeling reagents with dehydrogenases. These derivatives were developed to alkylate nucleophilic groups located at active sites of dehydrogenases; however, differences have been observed in their behavior with different dehydrogenases. For example, the 3-bromoacetylpyridine analogs in which the pyridine ribose has been substituted by alkyl groups [145,147] and 3-chloroacetylpyridine adenine dinucleotide do not inactivate lactate dehydrogenases from various sources. However, when the bromoacetyl group is in the 4-position as in 3-(4-bromoacetylpyridinio)propyladenosine pyrophosphate, selective inactivation of pig heart lactate dehydrogenase was observed [148]. The pH profile of this inactivation reaction suggested selective modification of an essential histidine residue of the enzyme.

Yeast alcohol dehydrogenase, due to the positioning of one of its essential sulfhydryl groups appears to be extremely sensitive to alkylation by haloacetyl analogs of NAD. Nicotinamide 5-bromoacetyl-4-methylimidazole dinucleotide [131], 3-(4-bromoacetylpyridinio)- and 3-(3-bromoacetylpyridinio)propyladenosine diphosphate inactivate yeast alcohol dehydrogenase by covalent modification of cysteine-43 of this enzyme [233]. Using ^{14}C-labeled analogs, complete inactivation of yeast alcohol dehydrogenase was demonstrated to involve the incorporation of 4 moles of reagent per 150,000 MW or one per catalytic site [234–236]. 4-(3-Bromoacetylpyridinio)butyladenosine diphosphate [145] and 3-chloroacetylpyridine adenine dinucleotide [25] also inactivate yeast alcohol dehydrogenase. With the exception of 3-(3-bromoacetylpyridinio)propyladenosine phosphate, these halomethyl ketone analogs have been demonstrated to inactivate horse liver alcohol dehydrogenase in a similar site-labeling fashion. Considerable evidence exists indicating yeast and horse liver alcohol dehydrogenases are homologous proteins sharing many properties in common. Each enzyme has two closely associated essential sulfhydryl groups located in or nearby the active site region, presumably serving as ligands for catalytic zinc atoms [237–243]. These groups have been identified as cysteine-43 and cysteine-153 of the yeast enzyme [238] and cysteine-46 and cysteine-174 of the horse liver enzyme [243]. Nicotinamide 4-methyl-5-bromoacetylimidazole dinucleotide selectively modifies cysteine-43 of the yeast enzyme; however, site-labeling of horse liver alcohol dehydrogenase by this reagent involves the modification of cysteine-174 instead of the homologous cysteine-46 [131]. The same residue labeling was observed in the inactivation of these two dehydrogenases by 3-(4-bromoacetylpyridinio)propyl adenosine diphosphate; however, inactivation of the two enzymes by 4-(3-bromoacetylpyridinio)butyladenosine diphosphate resulted in the modification of cysteine-43 of the yeast enzyme and the homologous cysteine-46 of the liver enzyme [244]. In addition, 3-(3-bromacetylpyridinio)propyladenosine diphos-

phate which selectively modifies cysteine-43 of the yeast enzyme failed to modify either of the two cysteine residues of horse liver alcohol dehydrogenase. Therefore, a comparison of site-labeling by pyridine nucleotide analogs containing bromacetyl groups in different positions of the dinucleotide molecule can provide new information concerning the positioning of functional groups at the active sites of dehydrogenases. Although the above observations confirm X-ray crystallographic data that two closely associated sulfhydryl groups exist in the active sites of both yeast and horse liver alcohol dehydrogenase, the differences observed in the patterns of site labeling clearly indicate that the active centers of these related enzymes are not identical [*244*]. The successful application of these pyridine nucleotide analogs for the study of alcohol dehydrogenases has prompted similar studies with these and other haloacetyl derivatives for the investigation of numerous other dehydrogenases [*71,132,149,232,235,245-250*].

A different approach to site labeling was developed by Chan and Anderson [*21*] based on the azo coupling reactions of diazotized 3-aminopyridine adenine dinucleotide [*20*]. The 3-aminopyridine analog of NAD can be diazotized in nitrous acid and the resulting diazonium chloride was demonstrated to azo couple at low pH with *N*-1-naphthylethylenediamine to form an azo dye absorbing maximally at 510 nm. It was anticipated that this diazotized dinucleotide analog would be selectively bound at enzyme NAD binding sites and thereby label active site tyrosyl and histidinyl residues through azo coupling reactions. Such reactions can be demonstrated with diazotized 3-aminopyridine adenine dinucleotide and model tyrosine and histidine derivatives. Unfortunately, these azo coupling reactions were observed not to occur above pH 3.5, an observation attributed to the facile conversion of the diazonium group to a diazohydroxide or diazotate promoted at these lower pHs by the electron-withdrawing properties of the pyridinium ring. Since site-labeling experiments with dehydrogenases and other NAD-requiring enzymes would normally require incubation of the enzymes with the diazotized reagent in the neutral pH region, modification of tyrosine and histidine residues would not be expected. Although these limitations were somewhat discouraging, interest in the application of diazotized 3-aminopyridine adenine dinucleotide as a site-labeling reagent was renewed by the observation that this reagent at 200 μM concentration rapidly inactivated yeast alcohol dehydrogenase ($t_{\frac{1}{2}}$ 2.5 min) under the mild conditions of pH 7 and 0°–5°C [*21*]. That site labeling was truly involved was demonstrated by a variety of procedures. Yeast alcohol dehydrogenase is a tetrameric protein composed of four identical subunits each containing an independently functioning active site. After complete inactivation of the enzyme by diazotized 3-aminopyridine adenine dinucleotide and removal of the reagent by extensive dialysis, the modified enzyme was demonstrated to contain four diazotized 3-aminopyridine adenine dinucleotide residues per 150,000 MW or one per active site of the enzyme. These calculations were based on the additional 260 nm absorbance determined for the modified and completely inactivated enzyme. Since modification of

tyrosine and histidine residues appeared
tion was performed, covalent modific
investigated. DTNB titrations of the e
vealed the loss of four sulfhydryl group
during the inactivation process. Since
enzyme was indicated, the spectral prop
interpreted to indicate the initial forma
sulfhydryls. Acid hydrolysis of the mo
ment of these derivatives to thioethe
3-pyridylcysteine was determined th
stoichiometry inherent in these analyse
one sulfhydryl group per NAD bindin
altered. The binding of NAD effectivel
tion by diazotized 3-aminopyridine ade
enzyme with 200 μM diazotized 3-a
phate did not result in any observable in
inactivation by the phosphorylated anal
specificity of yeast alcohol dehydroge
of the enzyme by the diazotized NAD
known which of the two essential cy
modified by diazotized 3-aminopyridin

Diazotized 3-aminopyridine adenine
a site-labeling reagent for rabbit muscl
Rapid inactivation of this enzyme was
of the reagent at low temperatures and
to the specific modification of one sulf
protection was afforded by the binding
were the observations that diazotized 3
not effectively inactivate bovine lactate
mitochondrial malate dehydrogenase.
expected since this enzyme has been de
reagents at neutral pH. Coenzym
3-aminopyridine adenine dinucleotide
the coenzyme binding site. The abser
zyme under conditions where the ana
hydryl group not to be located near th
tions were in obvious accord with X-r
reactive sulfhydryl groups of lactate
catalytic sites of the enzyme [*252*]
3-aminopyridine adenine dinucleotide
locate sulfhydryl groups at the active
nucleotide-requiring enzymes; howev
ferences observed in the site labeling

derson

e-43 of the yeast enzyme failed to of horse liver alcohol dehydrogenase. pyridine nucleotide analogs contain- ns of the dinucleotide molecule can sitioning of functional groups at the he above observations confirm X-ray ciated sulfhydryl groups exist in the cohol dehydrogenase, the differences arly indicate that the active centers of]. The successful application of these f alcohol dehydrogenases has promp- oacetyl derivatives for the investiga- *1,132,149,232,235,245-250*].

is developed by Chan and Anderson f diazotized 3-aminopyridine adenine nalog of NAD can be diazotized in oride was demonstrated to azo couple nine to form an azo dye absorbing at this diazotized dinucleotide analog AD binding sites and thereby label hrough azo coupling reactions. Such iazotized 3-aminopyridine adenine ine derivatives. Unfortunately, these occur above pH 3.5, an observation zonium group to a diazohydroxide or ie electron-withdrawing properties of periments with dehydrogenases and lly require incubation of the enzymes region, modification of tyrosine and lthough these limitations were some- ution of diazotized 3-aminopyridine ent was renewed by the observation rapidly inactivated yeast alcohol de- onditions of pH 7 and 0°-5°C [*21*]. nonstrated by a variety of procedures. protein composed of four identical functioning active site. After com- iazotized 3-aminopyridine adenine by extensive dialysis, the modified diazotized 3-aminopyridine adenine one per active site of the enzyme. onal 260 nm absorbance determined ed enzyme. Since modification of tyrosine and histidine residues appeared unlikely due to the pH at which inactivation was performed, covalent modification of other amino acid residues was investigated. DTNB titrations of the enzyme before and after inactivation revealed the loss of four sulfhydryl groups per 150,000 MW or one per active site during the inactivation process. Since modification of cysteinyl residues of the enzyme was indicated, the spectral properties of the inactivated enzyme could be interpreted to indicate the initial formation of diazomerceptides with active site sulfhydryls. Acid hydrolysis of the modified enzyme resulted in the rearrangement of these derivatives to thioethers and the release of 4 moles of *S*-3-pyridylcysteine was determined through amino acid analysis [*21*]. The stoichiometry inherent in these analyses indicated the covalent modification of one sulfhydryl group per NAD binding site with no other amino acid residues altered. The binding of NAD effectively protected the enzyme against inactivation by diazotized 3-aminopyridine adenine dinucleotide. Incubation of the yeast enzyme with 200 μM diazotized 3-aminopyridine adenine dinucleotide phosphate did not result in any observable inactivation of the enzyme. The absence of inactivation by the phosphorylated analog is consistent with the known coenzyme specificity of yeast alcohol dehydrogenase and further supports the inactivation of the enzyme by the diazotized NAD analog as a site-directed process. It is not known which of the two essential cysteine residues of the yeast enzyme is modified by diazotized 3-aminopyridine adenine dinucleotide.

Diazotized 3-aminopyridine adenine dinucleotide was also demonstrated to be a site-labeling reagent for rabbit muscle glycerophosphate dehydrogenase [*251*]. Rapid inactivation of this enzyme was observed with micromolar concentrations of the reagent at low temperatures and neutral pH. Inactivation was again related to the specific modification of one sulfhydryl group per active site and effective protection was afforded by the binding of NAD. Included in these studies [*251*] were the observations that diazotized 3-aminopyridine adenine dinucleotide does not effectively inactivate bovine lactate dehydrogenase (M_4 isozyme) and bovine mitochondrial malate dehydrogenase. Inactivation of lactate dehydrogenase was expected since this enzyme has been demonstrated to be inactivated by sulfhydryl reagents at neutral pH. Coenzyme-competitive inhibition by diazotized 3-aminopyridine adenine dinucleotide indicated selective binding of the analog at the coenzyme binding site. The absence of irreversible inactivation of the enzyme under conditions where the analog is bound suggested the essential sulfhydryl group not to be located near the coenzyme binding site. These observations were in obvious accord with X-ray crystallographic data indicating that the reactive sulfhydryl groups of lactate dehydrogenase are not located near the catalytic sites of the enzyme [*252*]. It has been suggested that diazotized 3-aminopyridine adenine dinucleotide can serve as an experimental probe to locate sulfhydryl groups at the active sites of dehydrogenases and other pyridine nucleotide-requiring enzymes; however, the lesson learned from the subtle differences observed in the site labeling of alcohol dehydrogenases by bromoacetyl

derivatives would indicate that proper positioning of the bound dinucleotide analog can play a significant role in whether or not an existing sulfhydryl becomes modified. The selective binding of the diazotized analog and the absence of irreversible inactivation cannot be directly interpreted to indicate the absence of sulfhydryl groups in the active site region. In this respect, diazotized 3-aminopyridine adenine dinucleotide was observed to be a reversible coenzyme-competitive inhibitor of chicken liver 3-phosphoglycerate dehydrogenase but showed no irreversible inactivation of the enzyme [*179*]. That sulfhydryl groups exist in the active site region of this enzyme was suggested by studies of maleimide inactivation of the enzyme [*179*]; however, properties of this inactivation process indicate the sulfhydryl group(s) to be more closely associated with the substrate-binding site.

The NADP analog, 3-aminopyridine adenine dinucleotide phosphate, has been synthesized and shown to have properties similar to those observed with the NAD analog [*200*]. The diazotized form of the NADP analog was demonstrated to be a coenzyme-competitive inhibitor of yeast glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase but no irreversible inactivation was observed [*200*] at these binding concentrations. This diazotized analog was however effectively applied to the study of *Neurospora crassa* nitrate reductase [*253*]. The diazotized NADP analog selectively inactivated all NADPH-dependent activities of the reductase, such as the NADPH-nitrate reductase and NADPH-cytochrome *c* reductase activities. The reduced methyl viologen and reduced FAD-nitrate reductase activities were unaffected. Inactivation of the enzyme was demonstrated to be related to the modification of sulfhydryl groups. Through studies of an unexpected FAD protection of this enzyme, these investigators [*253*] learned for the first time that this enzyme contained an oxidation-reduction-active disulfide and should be classified with other disulfide oxidases. Electron transfer reactions catalyzed by this enzyme complex can be more effectively studied by first selectively inactivating electron transfer reactions initiated by NADPH.

With respect to the need for proper positioning of site-labeling pyridine nucleotide analogs, it is of interest that diazotized 3-aminopyridine adenine dinucleotide phosphate does not inactivate yeast glutathione reductase but does serve as an effective coenzyme-competitive inhibitor [*254*]. This is of special interest since yeast glutathione reductase is a disulfide oxidase and the sulfhydryls of the reduced enzyme are intimately involved in the catalytic process and show a very high reactivity toward sulfhydryl-modifying reagents [*254*].

C. *In Vivo* Studies

Evidence has been presented for the *in vivo* formation of pyridine nucleotide analogs [*41,92,255-261*]. One explanation for the pharmacologic action of pyridine bases involves the formation of NAD and NADP analogs in tissues through transglycosidation reactions an analogs with various pyridine nucleotid made to take advantage of these reaction tic agents. Upon the injection of 3-ac workers [*255*] reported the formation of brain, and spleen. In the livers of 3-acetylpyridine resulted in a fourfold i 3-acetylpyridine analog detected. It was ified in the liver by conversion to nic increased production of NAD. The absen extrahepatic tissues favors the ac 3-acetylpyridine and concomitant forma interest was the observation that the high neoplastic tissue after the injection of various strains of tumors [*255,256*]. The accompanied by a decrease in the nicotinamide was administered with 3-a the amount of analog formed was o nicotinamide was correlated with th nicotinamide of analog synthesis catalyze The formation of coenzyme analogs in profound effect on cellular metabolism s siderably from NAD and NADP in their l pyridine nucleotide-requiring enzymes. formation of 3-acetylpyridine adenine di of rats injected with 3-acetylpyridine.

6-Aminonicotinamide has been demo showing activity against the Walker adenocarcinomas [*264*], and several hu This antimetabolite has also been used st [*268,269*]. 6-Aminonicotinamide aden through the NAD glycohydrolase-catal [*41,92,270*]. and isolated from tissues c [*41,92,256*]. The *in vivo* effects of 6-am the formation of NAD and NADP analc analogs to function in hydride transfer [*273*] demonstrated 6-aminonicotinamide potent inhibitor of rat brain 6-phosphogl [*272,275*] discuss the effects of 6-amino pathway in light of the above inhibitic 6-phosphogluconate in brains of rats treat level of 6-phosphogluconate dehydrogen effectiveness of 6-aminonicotinamide in

through transglycosidation reactions and the subsequent interactions of these analogs with various pyridine nucleotide-requiring enzymes. Efforts have been made to take advantage of these reactions in the development of chemotherapeutic agents. Upon the injection of 3-acetylpyridine in mice, Kaplan and coworkers [255] reported the formation of 3-acetylpyridine adenine dinucleotide in brain and spleen. In the livers of these animals the administration of 3-acetylpyridine resulted in a fourfold increase in NAD concentration with no 3-acetylpyridine analog detected. It was suggested that 3-acetylpyridine is detoxified in the liver by conversion to nicotinic acid or nicotinamide leading to increased production of NAD. The absence of such detoxification mechanisms in extrahepatic tissues favors the accumulation of the antimetabolite, 3-acetylpyridine and concomitant formation of the coenzyme analog. Of further interest was the observation that the highest concentration of analog was found in neoplastic tissue after the injection of 3-acetylpyridine into mice containing various strains of tumors [255,256]. The formation of analog in tumor tissue was accompanied by a decrease in the tissue concentration of NAD. When nicotinamide was administered with 3-acetylpyridine, a significant decrease in the amount of analog formed was observed. This *in vivo* protection by nicotinamide was correlated with the observed *in vitro* inhibition by nicotinamide of analog synthesis catalyzed by mouse brain NAD glycohydrolase. The formation of coenzyme analogs in tissues would be expected to have a profound effect on cellular metabolism since coenzyme analogs can differ considerably from NAD and NADP in their behavior with dehydrogenases and other pyridine nucleotide-requiring enzymes. Brunnemann *et al.* [258] reported the formation of 3-acetylpyridine adenine dinucleotide phosphate in the brain tissue of rats injected with 3-acetylpyridine.

6-Aminonicotinamide has been demonstrated to be an antineoplastic agent showing activity against the Walker carcinoma 256 [262,263], mammary adenocarcinomas [264], and several human malignant neoplasms [265–267]. This antimetabolite has also been used successfully in the treatment of psoriasis [268,269]. 6-Aminonicotinamide adenine dinucleotide has been prepared through the NAD glycohydrolase-catalyzed pyridine base-exchange reaction [41,92,270], and isolated from tissues of 6-aminonicotinamide-treated animals [41,92,256]. The *in vivo* effects of 6-aminonicotinamide have been attributed to the formation of NAD and NADP analogs in tissues and the inability of these analogs to function in hydride transfer reactions [41,271–274]. Lange *et al.* [273] demonstrated 6-aminonicotinamide adenine dinucleotide phosphate to be a potent inhibitor of rat brain 6-phosphogluconate dehydrogenase. Herken *et al.* [272,275] discuss the effects of 6-aminonicotinamide on the pentose phosphate pathway in light of the above inhibition and the observed accumulation of 6-phosphogluconate in brains of rats treated with 6-aminonicotinamide. Since the level of 6-phosphogluconate dehydrogenase increases in psoriasis [276,277], the effectiveness of 6-aminonicotinamide in treating psoriatic lesions [268] was

likewise suggested to involve in part the inhibition of this enzyme following the *in vivo* formation of 6-aminonicotinamide adenine dinucleotide phosphate. The usefulness of 6-aminonicotinamide as an antineoplastic agent or a chemotherapeutic agent for the treatment of skin disorders such as psoriasis is limited by serious toxic effects on the central nervous system [*265*]. These effects are manifested by motor weakness, limb paralysis, coma, and death [*278*]. Teratogenic effects of 6-aminonicotinamide in animals have also been reported [*279*]. Toxic and teratogenic effects have also been observed on administration of 3-acetylpyridine to animals [*256*], and although specific effects differ from those associated with 6-aminonicotinamide, dramatic neurological changes have been observed [*280,281*].

D. Clinical Studies

Analysis of the concentrations of dehydrogenases in blood serum has been used effectively for clinical diagnosis of disease states. Changes in the properties of serum lactate dehydrogenases were recognized early as an indicator of myocardial infarction [*282*]. Since lactate dehydrogenases are tetrameric and composed of kinetically different heart type (H) and muscle type (M) subunits, kinetic properties of a mixture of the five possible isozymes can be related to specific subunit composition. Changes in the isozyme patterns of serum lactate dehydrogenase serve as a sensitive indicator of specific tissue damage [*282–284*]. Several disease states are known to alter both lactate dehydrogenase concentration and isozyme patterns of serum [*285*]. Isozyme patterns of serum are routinely determined by electrophoretic methods which can be time-consuming. Methods utilizing more rapid kinetic analysis of both total lactate dehydrogenase concentration as well as the relative concentration of H and M subunits have been reported [*151,286*]. Bishop *et al.* [*286*] described a stopped-flow kinetic method that differentiates M_4 and H_4 isozymes on the basis of differing degrees of inhibition by high concentrations of pyruvate. These differences were accentuated by using 3-acetylpyridine adenine dinucleotide in place of the natural coenzyme. This method was purported to determine serum lactate dehydrogenase subunit composition more accurately than electrophoretic procedures and has been suggested as a rapid diagnostic tool for clinical medicine. These authors successfully demonstrated the application of this method in monitoring liver damage in patients.

Another method utilizing pyridine nucleotide analogs for clinical diagnosis of lactate dehydrogenase isozyme patterns has been reported by Minato *et al.* [*151*]. These authors recognized that there were significant differences in initial velocities catalyzed by H_4 and M_4 isozymes when one compared reaction mixtures containing 530 m*M* lactate and NAD and those containing 13 m*M* lactate and the NAD analog, thionicotinamide xanthine dinucleotide. The ratio of initial

velocities obtained with high lactate a
low lactate and thionicotinamide xanth
ratio. The *N/T* values for porcine H_4 a
0.49 and 9.33, respectively. The *N/T* v
calculated from subunit compositions.
agree well with experimental results
isozymes were assayed in the same m
tained for the H_4 isozyme and 6.66–7.5
exhibited an *N/T* ratio of 1.32. Myocar
however, hepatic diseases were accomp
value. These authors [*151*] indicated
important for clinical diagnosis and to
electrophoretic procedures.

Another diagnostic method of note i
analog for the study of alcohol dehyc
observed that an assay system for alcoh
adenine dinucleotide and amyl alcohol
assay system employing NAD and eth
diagnostic method based on the initial
dinucleotide reduction with amyl alcoh
velocities with the two substrates wer
dehydrogenase activity is almost exclus
systems [*283,289*], changes in the *A/E* r
damage. When the alcohol dehydrogena
fraction of the liver leaks into the bloo
A/E ratio was significantly elevated. I
active cirrhosis, and subacute hepatitis.
These authors suggest that the measure
provide considerable diagnostic inform
in chronic liver injury.

E. Evolution of Dehydrogenases

In early studies of pyridine nucleoti
dehydrogenases catalyzing the same re
could behave quite differently with
analogs. For example, the rate of
dinucleotide as catalyzed by yeast alcol
observed for NAD reduction [*28*], whe
alcohol dehydrogenase, rates of analog
tion were observed. Similar large di
3-acetylpyridine adenine dinucle

velocities obtained with high lactate and NAD divided by those obtained with low lactate and thionicotinamide xanthine dinucleotide was defined as the *N/T* ratio. The *N/T* values for porcine H_4 and M_4 isozymes were determined to be 0.49 and 9.33, respectively. The *N/T* values for the other three isozymes can be calculated from subunit compositions. The calculated values were observed to agree well with experimental results. When human lactate dehydrogenase isozymes were assayed in the same manner, *N/T* values of 1.0–1.57 were obtained for the H_4 isozyme and 6.66–7.5 for the M_4 isozyme. Normal human sera exhibited an *N/T* ratio of 1.32. Myocardial infarction did not alter the *N/T* ratio; however, hepatic diseases were accompanied by a significant increase in the *N/T* value. These authors [*151*] indicated the measurement of *N/T* values to be important for clinical diagnosis and to provide an advantage over conventional electrophoretic procedures.

Another diagnostic method of note involves the use of a pyridine nucleotide analog for the study of alcohol dehydrogenase [*287*]. Tamoaki *et al.* [*288*] observed that an assay system for alcohol dehydrogenase using thionicotinamide adenine dinucleotide and amyl alcohol was more sensitive than the conventional assay system employing NAD and ethanol. Fujisawa *et al.* [*287*] developed a diagnostic method based on the initial velocities of thionicotinamide adenine dinucleotide reduction with amyl alcohol and ethanol as substrates. The initial velocities with the two substrates were reported as *A/E* ratios. Since alcohol dehydrogenase activity is almost exclusively confined to the liver in mammalian systems [*283,289*], changes in the *A/E* ratios of serum can be used to detect liver damage. When the alcohol dehydrogenase activity associated with the particulate fraction of the liver leaks into the bloodstream as a result of liver damage, the *A/E* ratio was significantly elevated. In cases of chronic aggressive hepatitis, active cirrhosis, and subacute hepatitis, a relatively high *A/E* ratio was observed. These authors suggest that the measurement of *A/E* ratios of patients' sera could provide considerable diagnostic information concerning liver damage especially in chronic liver injury.

E. Evolution of Dehydrogenases

In early studies of pyridine nucleotide analogs [*28,64*] it was observed that dehydrogenases catalyzing the same reaction but isolated from different sources could behave quite differently with respect to the utilization of coenzyme analogs. For example, the rate of reduction of 3-acetylpyridine adenine dinucleotide as catalyzed by yeast alcohol dehydrogenase was one-tenth the rate observed for NAD reduction [*28*], whereas, in reactions catalyzed by horse liver alcohol dehydrogenase, rates of analog reduction ten times those of NAD reduction were observed. Similar large differences in the rates of reduction of 3-acetylpyridine adenine dinucleotide, pyridine-3-aldehyde adenine

dinucleotide, and NAD were reported in reactions catalyzed by beef heart and rabbit muscle lactate dehydrogenases [*28*]. These same alcohol and lactate dehydrogenases were also observed to behave quite differently with thionicotinamide adenine dinucleotide [*64*]. Therefore, one can distinguish common substrate dehydrogenases isolated from different sources by comparing rates of reduction of NAD and various analogs. Differences in the analog ratios obtained can be accentuated further by incorporating into the evaluation process other kinetic properties of the enzymes involved. For example, lactate dehydrogenases from different sources show different relationships with respect to substrate saturation and/or substrate inhibition by lactate and pyruvate. Therefore, if one studies the reduction of NAD and NAD analogs in the presence of high and low lactate concentrations, one can establish the proper analog ratios that will effectively distinguish lactate dehydrogenases from different sources. For example, the rates of reduction of 3-acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide at low lactate concentrations, catalyzed by heart muscle and skeletal muscle lactate dehydrogenases from different mammals, provided the data necessary for the comparison of these types of enzymes [*65,290*]. The analog ratios obtained in this fashion provided the interesting observation that the heart lactate dehydrogenases from eight mammalian species were more closely related than were the heart and skeletal muscle lactate dehydrogenases from any given species. These and related studies suggested the possibility that catalytic identification of enzymes could be of value in phylogenetic and taxonomic studies. One can classify organisms on the basis of the kinetic properties of lactate dehydrogenases as well as those of other dehydrogenases. Kaplan *et al.* [*290*] demonstrated through analog ratios that the lactate dehydrogenases from a number of crustaceans exhibited common kinetic properties. The horseshoe crab which is related to the arachnids was observed to have a lactate dehydrogenase with kinetic properties closely related to those of scorpions, spiders, and tarantulas, members of the same subphylum.

The use of analog ratios to determine specific properties and heterogeneity of dehydrogenases can be extended to other types of dehydrogenases. Malate dehydrogenases are of special interest due to a wider distribution of these enzymes in different life forms. Kaplan and Ciotti [*28*] have reported analog ratios for a large number of bacterial malate dehydrogenases and suggest an importance for this experimental approach for the classification of microorganisms.

VI. CONCLUDING REMARKS

The early studies of Kaplan and co-workers in the mid 1950s provided the basis for a new and exciting area of biochemical research. The demonstration that

analogs of the pyridine nucleotide coenzy
through the pyridine base-exchange reactio
provided the impetus for further studies re
variety of pyridine nucleotide analogs tha
important experimental tools for the inves
pyridine nucleotide-dependent enzymes. T
and co-workers in Germany on the diffi
pyridine nucleotide analogs effectively cor
viding a host of analogs that could not be s
importance of pyridine nucleotide analogs i
documented by simply considering the ove
coenzymes in oxidation–reduction reactio
recent implication of pyridine nucleotide c
reactions such as ADP-ribosylation and p
even greater importance for these compou
tion. As new reactions of pyridine nucleoti
derivatives of the dinucleotides soon follow
in the synthesis of fluorescent, spin-label
dinucleotides and continued efforts to desi
chromatography. Obvious differences in t
enzymes should continue to challenge rese
quired to effectively study these enzymes.
have been used successfully in enzyme studi
should be prepared. In the same sense, on
analog of NAD to be prepared since the cor
been reported. Missing from the long list of
are derivatives in which the pyrophosphate
to prevent hydrolysis in reactions catalyzed
phosphodiesterases. Dinucleotides with nitr
of the molecule would provide interesting c
of enzymes. Further studies are needed to
tuted pyridine analogs with some attention
hindered by substitution in the 5- and 6-p
pharmacological effects of 6-substituted nic
the analogs containing these pyridine base
increased investigation of the *in vivo* form
and the ultimate effects of these compoun
with the existence of more sophisticated an
high performance liquid chromatography, a
analogs should be possible and a more conv
cal structures of these compounds should b

in reactions catalyzed by beef heart and
28]. These same alcohol and lactate de-
to behave quite differently with
[64]. Therefore, one can distinguish
ued from different sources by comparing
analogs. Differences in the analog ratios
incorporating into the evaluation process
s involved. For example, lactate dehy-
v different relationships with respect to
ibition by lactate and pyruvate. There-
D and NAD analogs in the presence of
e can establish the proper analog ratios
dehydrogenases from different sources.
-acetylpyridine adenine dinucleotide and
low lactate concentrations, catalyzed by
e dehydrogenases from different mam-
e comparison of these types of enzymes
in this fashion provided the interesting
ogenases from eight mammalian species
: heart and skeletal muscle lactate dehy-
e and related studies suggested the possi-
ymes could be of value in phylogenetic
y organisms on the basis of the kinetic
well as those of other dehydrogenases.
gh analog ratios that the lactate dehy-
as exhibited common kinetic properties.
the arachnids was observed to have a
erties closely related to those of scorp-
of the same subphylum.
specific properties and heterogeneity of
er types of dehydrogenases. Malate de-
to a wider distribution of these enzymes
i [28] have reported analog ratios for a
ogenases and suggest an importance for
ification of microorganisms.

workers in the mid 1950s provided the
emical research. The demonstration that

analogs of the pyridine nucleotide coenzymes could be prepared enzymatically through the pyridine base-exchange reaction catalyzed by mammalian NADases provided the impetus for further studies resulting ultimately in the synthesis of a variety of pyridine nucleotide analogs that have since proven to be extremely important experimental tools for the investigation of dehydrogenases and other pyridine nucleotide-dependent enzymes. The excellent studies of Woenckhaus and co-workers in Germany on the difficult task of chemically synthesizing pyridine nucleotide analogs effectively complemented Kaplan's studies by providing a host of analogs that could not be synthesized by enzymatic means. The importance of pyridine nucleotide analogs in biochemical research can be readily documented by simply considering the overall importance of pyridine nucleotide coenzymes in oxidation–reduction reactions of metabolic pathways. The more recent implication of pyridine nucleotide coenzymes in non-oxidation–reduction reactions such as ADP-ribosylation and polyADP-ribose formation predicts an even greater importance for these compounds in future biochemical investigation. As new reactions of pyridine nucleotides are discovered, the need for new derivatives of the dinucleotides soon follows. One can expect a continued interest in the synthesis of fluorescent, spin-labeled, and site-labeling derivatives of dinucleotides and continued efforts to design derivatives to be used in affinity chromatography. Obvious differences in the NAD and NADP binding sites of enzymes should continue to challenge researchers to design the derivatives required to effectively study these enzymes. In several cases where NAD analogs have been used successfully in enzyme studies, the corresponding NADP analogs should be prepared. In the same sense, one can expect the selenonicotinamide analog of NAD to be prepared since the corresponding NADP analog has already been reported. Missing from the long list of available pyridine nucleotide analogs are derivatives in which the pyrophosphate group has been sufficiently modified to prevent hydrolysis in reactions catalyzed by nucleotide pyrophosphatases and phosphodiesterases. Dinucleotides with nitrogen or methylene bridges in this part of the molecule would provide interesting derivatives for the study of a number of enzymes. Further studies are needed to improve the preparation of disubstituted pyridine analogs with some attention paid to those presumably sterically hindered by substitution in the 5- and 6-position of the pyridine ring. Known pharmacological effects of 6-substituted nicotinamides warrant further studies of the analogs containing these pyridine bases. In general, one would hope for increased investigation of the *in vivo* formation of pyridine nucleotide analogs and the ultimate effects of these compounds on cellular metabolism. Finally, with the existence of more sophisticated analytical techniques such as NMR and high performance liquid chromatography, a greater purity of pyridine nucleotide analogs should be possible and a more convincing characterization of the chemical structures of these compounds should be provided.

ACKNOWLEDGMENT

The author acknowledges the generosity of Professors C. Woenckhaus and J. F. Biellmann in providing preprints and published materials and the aid received from Drs. W. G. Niehaus, Jr., E. M. Gregory, R. E. Dubler, and D. A. Yost in the discussion of the text material.

REFERENCES

1. Schlenk, F., Hellstrom, H., and von Euler, H. (1938). *Ber.* 71, 1471.
2. Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. (1952). *J. Biol. Chem.* 194, 579.
3. Hughes, N. A., Kenner, G. W., and Todd, A. R. (1957). *J. Chem. Soc.* 3733.
4. Woenckhaus, C. (1974). *Fortsch. Chem. Forsch.* 52, 209.
5. Shuster, L., Kaplan, N. O., and Stolzenbach, F. E. (1955). *J. Biol. Chem.* 215, 195.
6. Moffat, J. G., and Khorana, H. G. (1958). *J. Am. Chem. Soc.* 80, 3756.
7. Ikehara, M. (1960). *Chem. Pharm. Bull.* 8, 367.
8. Davoll, J., and Lowy, B. A. (1951). *J. Am. Chem. Soc.* 73, 1650.
9. Robins, M. J., and Robins, R. K. (1965). *J. Am. Chem. Soc.* 87, 4934.
10. Tener, G. M. (1961). *J. Am. Chem. Soc.* 83, 159.
11. Seita, T., Yamanchi, K., Kinoshita, M., and Imoto, M. (1972). *Bull. Chem. Soc. Jpn* 45, 926.
12. Jeck, R., Heik, P., and Woenckhaus, C. (1974). *FEBS Lett.* 42, 161.
13. Windmueller, H. G., and Kaplan, N. O. (1961). *J. Biol. Chem.* 236, 2716.
14. Lindberg, M., Larsson, P. O., and Mosbach, K. (1973). *Eur. J. Biochem.* 40, 187.
15. Lee, C-Y., Lappi, D. A., Wermuth, B., Everse, J., and Kaplan, N. O. (1974). *Arch. Biochem. Biophys.* 163, 561.
16. Lee, C-Y., and Kaplan, N. O. (1975). *Arch. Biochem. Biophys.* 168, 665.
17. Abdallah, M. A., Biellmann, J. F., Nordstrom, B., and Branden, C-I. (1975). *Eur. J. Biochem.* 50, 475.
18. Barrio, J. R., Secrist, J. A., III, and Leonard, N. J. (1972). *Proc. Natl. Acad. Sci. U.S.A.* 69, 2039.
19. Muramatsu, M., Urabe, I., Yamada, Y., and Okada, H. (1977). *Eur. J. Biochem.* 80, 111.
20. Fisher, T. L., Vercellotti, S. V., and Anderson, B. M. (1973). *J. Biol. Chem.* 248, 4293.
21. Chan, J. K., and Anderson, B. M. (1975). *J. Biol. Chem.* 250, 67.
22. Abdallah, M. A., Biellmann, J. F., Samama, J. P., and Wrixon, A. D. (1976). *Eur. J. Biochem.* 64, 351.
23. Anderson, B. M., Ciotti, M. M., and Kaplan, N. O. (1959). *J. Biol. Chem.* 221, 1219.
24. Biellmann, J. F., and Jung, M. J. (1970). *FEBS Lett.* 7, 199.
25. Biellmann, J. F., Branlant, G., Foucaud, B. Y., and Jung, M. J. (1974). *FEBS Lett.* 40, 29.
26. Biellmann, J. F., and Jung, M. J. (1971). *Eur. J. Biochem.* 19, 130.
27. Kaplan, N. O., and Ciotti, M. M. (1956). *J. Biol. Chem.* 221, 823.
28. Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1956). *J. Biol. Chem.* 221, 833.
29. Abdallah, M. A., Andre, J. J., and Biellmann, J. F. (1977). *Bioorg. Chem.* 6, 157.
30. Pfleiderer, G., Woenckhaus, C., Scholz, K., and Feller, H. (1964). *Liebigs Ann. Chem.* 675, 205.
31. Woenckhaus, C., and Duchmann, H. (1975). *Z. Naturforsch.* 30c, 562.
32. Woenckhaus, C., and Jeck, R. (1971). *Z. Physiol. Chem.* 352, 1417.
33. Chen, S., and Guillory, R. J. (1977). *J. Biol. Chem.* 252, 8990.
34. Lamed, R., Levin, Y., and Wilchek, M. (1973). *Biochim. Biophys. Acta* 304, 231.

35. Zatman, L. J., Colowick, S. P., Kaplan, N. *Hosp.* 91, 211.
36. Zatman, L. J., Kaplan, N. O., and Colow
37. Zatman, L. J., Kaplan, N. O., Colowick, S 453.
38. Zatman, L. J., Kaplan, N. O., Colowick, S 467.
39. Kaplan, N. O., and Ciotti, M. M. (1956).
40. Walter, P., and Kaplan, N. O. (1963). *J.*
41. Dietrich, L. S., Friedland, I. M., and Kap
42. Brummemann, A., Coper, H., and Neuber 437.
43. Dickerman, H. W., San Pietro, A., and 230.
44. Swislocki, N. I., and Kaplan, N. O. (196
45. Swislocki, N. I., Kalish, M. L., Chasalow 242, 1089.
46. Schuber, F., Travo, P., and Pascal, M. (1
47. Iyer, P. K., and Jagannathan, V. (1976).
48. Kaplan, N. O. (1955). *Methods Enzymol.*
49. Green, S., and Bodansky, O. (1965). *J. I*
50. Schuber, F., Travo, P., and Pascal, M. (1
51. Yost, D. A., and Anderson, B. M., unpu
52. Yuan, J. H., and Anderson, B. M. (1971
53. Yuan, J. H., and Anderson, B. M. (1973
54. Anderson, B. M., and Fisher, T. L. (198
55. Yuan, J. H., Barnett, L. B., and Anderso
56. Pekala, P. H., and Anderson, B. M. (197
57. Bhattacharya, K. L. (1953). *J. Indian Ch*
58. Suzuki, T., Iizuka, K., and Murata, Y. (1
59. Tatsuki, T., Iwanaga, S., Oshima, G., an
60. Komberg, A. (1950). *J. Biol. Chem.* 182
61. Atkinson, M. A., Jackson, J. F., Morton, 196, 35.
62. Ward, D. C., Horn, T., and Reich, E. (1
63. Suhadolnik, R. J., Lennon, M. B., Uem *Biol. Chem.* 252, 4125.
64. Anderson, B. M., and Kaplan, N. O. (19
65. Kaplan, N. O., and Ciotti, M. M. (1961)
66. Lamborg, M. R., Stolzenbach, F. E., an
67. Biellmann, J. F., Samama, J. P., and Wi
68. Stein, A. M., Lee, J. K., Anderson, C. 1015.
69. Anderson, B. M., Anderson, C. D., Lee 1017.
70. Wallenfels, K., Gelfrich, M., and Kubowi
71. Biellmann, J. F., Branlant, G., Nicolas, J A. (1976). *Eur. J. Biochem.* 63, 477.
72. Samama, J. P., Zeppezauer, E., Biellmann 81, 403.

35. Zatman, L. J., Colowick, S. P., Kaplan, N. O., and Ciotti, M. M. (1952). *Bull. Johns Hopkins Hosp.* **91**, 211.
36. Zatman, L. J., Kaplan, N. O., and Colowick, S. P. (1953). *J. Biol. Chem.* **200**, 197.
37. Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. (1954). *J. Biol. Chem.* **209**, 453.
38. Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. (1954). *J. Biol. Chem.* **209**, 467.
39. Kaplan, N. O., and Ciotti, M. M. (1956). *J. Biol. Chem.* **221**, 823.
40. Walter, P., and Kaplan, N. O. (1963). *J. Biol. Chem.* **238**, 2823.
41. Dietrich, L. S., Friedland, I. M., and Kaplan, L. A. (1958). *J. Biol. Chem.* **233**, 964.
42. Brunnemann, A., Coper, H., and Neubert, D. (1964). *Arch. Exp. Pathol. Pharmakol.* **246**, 437.
43. Dickerman, H. W., San Pietro, A., and Kaplan, N. O. (1962). *Biochim. Biophys. Acta* **62**, 230.
44. Swislocki, N. I., and Kaplan, N. O. (1967). *J. Biol. Chem.* **242**, 1083.
45. Swislocki, N. I., Kalish, M. I., Chasalow, F. I., and Kaplan, N. O. (1967). *J. Biol. Chem.* **242**, 1089.
46. Schuber, F., Travo, P., and Pascal, M. (1976). *Eur. J. Biochem.* **69**, 593.
47. Iyer, P. K., and Jagannathan, V. (1976). *Indian J. Biochem. Biophys.* **13**, 197.
48. Kaplan, N. O. (1955). *Methods Enzymol.* **2**, p. 660.
49. Green, S., and Bodansky, O. (1965). *J. Biol. Chem.* **240**, 2574.
50. Schuber, F., Travo, P., and Pascal, M. (1979). *Bioorg. Chem.* **8**, 83.
51. Yost, D. A., and Anderson, B. M., unpublished results.
52. Yuan, J. H., and Anderson, B. M. (1971). *J. Biol. Chem.* **246**, 2111.
53. Yuan, J. H., and Anderson, B. M. (1973). *J. Biol. Chem.* **248**, 417.
54. Anderson, B. M., and Fisher, T. L. (1980). *Methods Enzymol.* **66**, 80.
55. Yuan, J. H., Barnett, L. B., and Anderson, B. M. (1972). *J. Biol. Chem.* **247**, 511.
56. Pekala, P. H., and Anderson, B. M. (1978). *J. Biol. Chem.* **253**, 7453.
57. Bhattacharya, K. L. (1953). *J. Indian Chem. Soc.* **30**, 685.
58. Suzuki, T., Iizuka, K., and Murata, Y. (1960). *J. Pharm. Soc. Jpn.* **80**, 868.
59. Tatsuki, T., Iwanaga, S., Oshima, G., and Suzuki, T. (1975). *Toxicon* **13**, 211.
60. Kornberg, A. (1950). *J. Biol. Chem.* **182**, 779.
61. Atkinson, M. A., Jackson, J. F., Morton, R. K., and Murray, A. W. (1962). *Nature (London)* **196**, 35.
62. Ward, D. C., Horn, T., and Reich, E. (1972). *J. Biol. Chem.* **247**, 4014.
63. Suhadolnik, R. J., Lennon, M. B., Uematsu, T., Monahan, J. E., and Baur, R. (1977). *J. Biol. Chem.* **252**, 4125.
64. Anderson, B. M., and Kaplan, N. O. (1959). *J. Biol. Chem.* **234**, 1226.
65. Kaplan, N. O., and Ciotti, M. M. (1961). *Ann. N.Y. Acad. Sci.* **94**, 701.
66. Lamborg, M. R., Stolzenbach, F. E., and Kaplan, N. O. (1958). *J. Biol. Chem.* **231**, 685.
67. Biellmann, J. F., Samama, J. P., and Wrixon, A. D. (1973). *Biochimie* **55**, 1469.
68. Stein, A. M., Lee, J. K., Anderson, C. D., and Anderson, B. M. (1963). *Biochemistry* **2**, 1015.
69. Anderson, B. M., Anderson, C. D., Lee, J. K., and Stein, A. M. (1963). *Biochemistry* **2**, 1017.
70. Wallenfels, K., Gellrich, M., and Kubowitz, F. (1959). *Justus Liebigs Ann. Chem.* **621**, 137.
71. Biellmann, J. F., Branlant, G., Nicolas, J. C., Pons, M., Descomps, B., and Crastes de Paulet, A. (1976). *Eur. J. Biochem.* **63**, 477.
72. Samama, J. P., Zeppezauer, E., Biellmann, J. F., and Branden, C. I. (1977). *Eur. J. Biochem.* **81**, 403.

73. Hixson, S. S., and Hixson, S. H. (1973). *Photochem. Photobiol.* **18**, 135.
74. Browne, D. T., Hixson, S. S., and Westheimer, F. H. (1971). *J. Biol. Chem.* **246**, 4677.
75. Masingale, R. E., and Skinner, C. G. (1975). *Arch. Biochem. Biophys.* **169**, 443.
76. Shen, W. C., and Van Vunakis, H. (1974). *Biochemistry* **13**, 5362.
77. Shen, W. C., and Van Vunakis, H. (1974). *Res. Commun. Chem. Pathol. Pharmacol.* **9**, 405.
78. Shen, W. C., Franke, J., and Van Vunakis, H. (1977). *Biochem. Pharmacol.* **26**, 1835.
79. Langer, R., and Glaser, L. (1974). *J. Biol. Chem.* **249**, 1126.
80. Muller, H., and Anderson, B. M., unpublished results.
81. Vorontsov, E. A., Lifshits, N. L., Mal'tsev, N. I., and Yakelov, V. A. (1976). *Biokhimiya (Moscow)* **41**, 248.
82. Sarma, R. H., Moore, M., and Kaplan, N. O. (1970). *Biochemistry* **9**, 549.
83. Sarma, R. H., Ross, V., and Kaplan, N. O. (1968). *Biochemistry* **7**, 3052.
84. Vorontsov, E. A., Mal'tsev, N. I., Shchors, E. I., Yakelov, V. A., and Glotov, B. O. (1974). *Biofizika* **19**, 626.
85. Vorontsov, E. A., Kalacheva, N. I., and Mal'tsev, N. I. (1979). *Bioorg. Khim.* **5**, 680.
86. Standring, D. N., and Knowles, J. R. (1980). *Biochemistry* **19**, 2811.
87. Van Eys, J. (1958). *J. Biol. Chem.* **233**, 1203.
88. Burton, R. M., and Kaplan, N. O. (1957). *Arch. Biochem. Biophys.* **70**, 107.
89. Burton, R. M., San Pietro, A., and Kaplan, N. O. (1957). *Arch. Biochem. Biophys.* **70**, 87.
90. Pfleiderer, G., Holbrook, T. T., and Wieland, T. (1963). *Biochem. Z.* **338**, 52.
91. Biellmann, J. F., and Hirth, C. G. (1975). *Eur. J. Biochem.* **56**, 557.
92. Johnson, W. J., and McColl, J. D. (1956). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **15**, 284.
93. Trommer, W. E., Blume, H., and Kapmeyer, H. (1976). *Justus Liebigs Ann. Chem.* 848.
94. Kapmeyer, H., Pfleiderer, G., and Trommer, W. E. (1976). *Biochemistry* **15**, 5024.
95. Grau, U., Kapmeyer, H., and Trommer, W. E. (1978). *Biochemistry* **17**, 4621.
96. Trommer, W. E., Kapmeyer, H., and Pfleiderer, G. (1975). *Z. Physiol. Chem.* **356**, 101.
97. Freyne, E. J., Esmans, E. L., Vanbroeckhoven, J. H., Lepoivre, J. A., and Alderweireldt, F. C. (1978). *Bull. Soc. Chim. Belg.* **87**, 801.
98. Gourlay, G. K., and Stock, B. H. (1978). *Biochem. Pharmacol.* **27**, 979.
99. Alivisatos, S. G. A., and Woolley, D. W. (1955). *J. Am. Chem. Soc.* **77**, 1065.
100. Alivisatos, S. G. A., Ungar, F., Luckas, L., and LaMantia, L. (1960). *J. Biol. Chem.* **235**, 1742.
101. Alivisatos, S. G. A., and LaMantia, L. (1960). *Biochem. Biophys. Res. Commun.* **2**, 164.
102. Nelson, J. A., Rose, L. M., and Bennett, L. L., Jr. (1977). *Cancer Res.* **37**, 182.
103. Ciotti, M. M., Humphreys, S. R., Venditti, J. M., Kaplan, N. O., and Goldin, A. (1960). *Cancer Res.* **20**, 1195.
104. Van Eys, J., and Kaplan, N. O. (1957). *J. Biol. Chem.* **228**, 305.
105. Pfleiderer, G., Hohnholz-Merz, E., and Gerlach, D. (1962). *Biochem. Z.* **336**, 371.
106. Leighton, S. B. (1957). Ph.D. Thesis, Johns Hopkins Univ., Baltimore, Maryland.
107. Danenberg, P. V., Danenberg, K. D., and Cleland, W. W. (1978). *J. Biol. Chem.* **253**, 5886.
108. Combs, G. H., and Rabin, B. R. (1974). *FEBS Lett.* **42**, 231.
109. Goebbeler, K. H., and Woenckhaus, C. (1966). *Justus Liebigs Ann. Chem.* **700**, 180.
110. Woenckhaus, C., and Jeck, R. (1970). *Justus Liebigs Ann. Chem.* **736**, 126.
111. Woenckhaus, C., Volz, M., and Pfleiderer, G. (1964). *Z. Naturforsch.* **19b**, 467.
112. Gohring, I., Wacker, A., and Pfleiderer, G. (1964). *Biochem. Z.* **339**, 514.
113. Craven, D. B., Harvey, M. J., and Dean, P. D. G. (1974). *FEBS Lett.* **38**, 320.
114. Trommer, W. E., Wenzel, H., and Pfleiderer, G. (1974). *Justus Liebigs Ann. Chem.* **8**, 1357.
115. Grenner, G., Schmidt, H. L., and Vokl, W. (1976). *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 887.
116. Koberstein, R. (1976). *Eur. J. Biochem.* **67**, 223.

117. Zappelli, P., Rossodivita, A., and Re, L.,
118. Mantovani, E., Settembri, L., and Zappel
119. Lappi, D. A., Evans, F. E., and Kaplan,
120. Fawcett, C. P., and Kaplan, N. O. (1962)
121. Woenckhaus, C., and Scherr, D. (1971).
122. Honjo, M., Furukawa, Y., Moriyama, H., 73.
123. Pfleiderer, G., Sann, E., and Ortanderl, F
124. Woenckhaus, C., and Scherr, D. (1973).
125. Woenckhaus, C. (1964). *Chem. Ber.* **97**,
126. Woenckhaus, C., and Pfleiderer, G. (1965
127. Woenckhaus, C., and Zumpe, P. (1968).
128. Woenckhaus, C., and Volz, M. H. (1966)
129. Scherr, D., Jeck, R., Berghäuser, J., and
130. Woenckhaus, C., Kaleja, R., and Heik, P.
131. Jornvall, H., Woenckhaus, C., and Johnsc
132. Johnscher, G., Woenckhaus, C., Nicolas, Paulet, A. (1976). *FEBS Lett.* **61**, 176.
133. Greenfield, J. C., Leonard, N. J., and Gu
134. Stein, A. M. (1971). *FEBS Lett.* **19**, 270.
135. Klenow, H., and Andersen, B. (1957). *Bi*
136. Jeck, R., and Wilhelm, G. (1973). *Justus*
137. Jeck, R., Woenckhaus, C., and Holy, A.
138. Chen, S., and Guillory, R. J. (1979). *J. B*
139. Pfleiderer, G., Sann, E., and Stock, A. (1
140. Siegel, J. M., Montgomery, G. A., and B 288.
141. van Eys, J., Ciotti, M. M., and Kaplan, N
142. Berariu, V., Jeck, R., and Woenckhaus, C
143. Weiner, H. (1969). *Biochemistry* **8**, 526.
144. Veinberg, A. Ya., Gracheva, I. N., and So 2341.
145. Jeck, R., Zumpe, P., and Woenckhaus, C.
146. Jeck, R. (1977). *Z. Naturforsch.* **32c**, 550.
147. Woenckhaus, C., Zoltobrocki, M., and Ber
148. Woenckhaus, C., Schattle, E., Jeck, R., a 559.
149. Jeck, R., and Woenckhaus, C. (1974). *Z.*
150. Mel'nikova, L. M., and Berezovskii, V. *M*
151. Minato, S., Tamaoki, H., and Mizushima,
152. Kaplan, N. O., Ciotti, M. M., Stolzenbach *Soc.* **77**, 815.
153. Schott, E., and Schlimme, E. (1978). *Z. P*
154. Leonard, N. J., and Laursen, R. A. (1965)
155. Wong, S. S. (1980). *Fed. Proc., Fed. Am.*
156. Freundenthal, R. I. (1980). *Methods Enzyn*
157. Rafter, G. W., and Colowick, S. P. (1957)
158. Dunn, M. F., and Hutchinson, J. S. (1973)
159. Priess, J., and Handler, P. (1958). *J. Biol.*
160. Hughes, D. E., and Williamson, D. H. (19

117. Zappelli, P., Rossodivita, A., and Re, L. (1975). *Eur. J. Biochem.* **54**, 475.
118. Mantovani, E., Settembri, L., and Zappelli, P. (1976). *Tetrahedron* **32**, 1761.
119. Lappi, D. A., Evans, F. E., and Kaplan, N. O. (1980). *Biochemistry* **19**, 3841.
120. Fawcett, C. P., and Kaplan, N. O. (1962). *J. Biol. Chem.* **237**, 1709.
121. Woenckhaus, C., and Scherr, D. (1971). *Z. Naturforsch.* **26b**, 106.
122. Honjo, M., Furukawa, Y., Moriyama, H., and Tamaka, K. (1962). *Chem. Pharm. Bull.* **10**, 73.
123. Pfleiderer, G., Sann, E., and Ortanderl, F. (1963). *Biochim. Biophys. Acta* **73**, 39.
124. Woenckhaus, C., and Scherr, D. (1973). *Z. Physiol. Chem.* **354**, 53.
125. Woenckhaus, C. (1964). *Chem. Ber.* **97**, 2439.
126. Woenckhaus, C., and Pfleiderer, G. (1965). *Biochem. Z.* **341**, 495.
127. Woenckhaus, C., and Zumpe, P. (1968). *Z. Naturforsch.* **23b**, 484.
128. Woenckhaus, C., and Volz, M. H. (1966). *Chem. Ber.* **99**, 1712.
129. Scherr, D., Jeck, R., Berghauser, J., and Woenckhaus, C. (1973). *Z. Naturforsch.* **28c**, 247.
130. Woenckhaus, C., Kaleja, R., and Heik, P. (1970). *Z. Naturforsch.* **25b**, 1252.
131. Jornvall, H., Woenckhaus, C., and Johnscher, G. (1975). *Eur. J. Biochem.* **53**, 71.
132. Johnscher, G., Woenckhaus, C., Nicolas, J. C., Pons, M., Descomps, B., and Crastes de Paulet, A. (1976). *FEBS Lett.* **61**, 176.
133. Greenfield, J. C., Leonard, N. J., and Gumport, R. I. (1975). *Biochemistry* **14**, 698.
134. Stein, A. M. (1971). *FEBS Lett.* **19**, 270.
135. Klenow, H., and Andersen, B. (1957). *Biochim. Biophys. Acta* **23**, 92.
136. Jeck, R., and Wilhelm, G. (1973). *Justus Liebigs Ann. Chem.* 531.
137. Jeck, R., Woenckhaus, C., and Holy, A. (1975). *Z. Naturforsch.* **30c**, 734.
138. Chen, S., and Guillory, R. J. (1979). *J. Biol. Chem.* **254**, 7220.
139. Pfleiderer, G., Sann, E., and Stock, A. (1960). *Chem. Ber.* **93**, 3083.
140. Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959). *Arch. Biochem. Biophys.* **82**, 288.
141. van Eys, J., Ciotti, M. M., and Kaplan, N. O. (1958). *J. Biol. Chem.* **231**, 571.
142. Berariu, V., Jeck, R., and Woenckhaus, C. (1978). *Justus Liebigs Ann. Chem.* 118.
143. Weiner, H. (1969). *Biochemistry* **8**, 526.
144. Veinberg, A. Ya., Gracheva, I. N., and Samokhvalov, G. I. (1972). *Zh. Obshch. Khim.* **42**, 2341.
145. Jeck, R., Zumpe, P., and Woenckhaus, C. (1973). *Justus Liebigs Ann. Chem.* 961.
146. Jeck, R. (1977). *Z. Naturforsch.* **32c**, 550.
147. Woenckhaus, C., Zoltobrocki, M., and Berghauser, J. (1970). *Z. Physiol. Chem.* **351**, 1441.
148. Woenckhaus, C., Schattle, E., Jeck, R., and Berghauser, J. (1972). *Z. Physiol. Chem.* **353**, 559.
149. Jeck, R., and Woenckhaus, C. (1974). *Z. Naturforsch.* **29c**, 180.
150. Mel'nikova, L. M., and Berezovskii, V. M. (1970). *Zh. Obsch. Khim.* **40**, 918.
151. Minato, S., Tamaoki, H., and Mizushima, H. (1976). *Clin. Chim. Acta* **69**, 243.
152. Kaplan, N. O., Ciotti, M. M., Stolzenbach, F. E., and Bachur, N. R. (1955). *J. Am. Chem. Soc.* **77**, 815.
153. Schott, E., and Schlimme, E. (1978). *Z. Physiol. Chem.* **359**, 1675.
154. Leonard, N. J., and Laursen, R. A. (1965). *Biochemistry* **4**, 365.
155. Wong, S. S. (1980). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **39**, 1698.
156. Freundenthal, R. I. (1980). *Methods Enzymol.* **66** (in press).
157. Rafter, G. W., and Colowick, S. P. (1957). *Methods Enzymol.* **3**, 887.
158. Dunn, M. F., and Hutchinson, J. S. (1973). *Biochemistry* **12**, 4882.
159. Priess, J., and Handler, P. (1958). *J. Biol. Chem.* **233**, 488.
160. Hughes, D. E., and Williamson, D. H. (1952). *Biochem. J.* **51**, 330.

161. Sundaram, T. K., Rajagopolan, K. V., Pichappa, C. V., and Sarma, P. S. (1960). *Biochem. J.* 77, 145.
162. Imsande, J. (1964). *Biochim. Biophys. Acta* 82, 445.
163. Kahn, V., and Blum, J. J. (1968). *J. Biol. Chem.* 243, 1441.
164. Chaykin, S. (1966). *Adv. Tracer Methodol.* 3, 1.
165. Friedman, H. C. (1971). *Methods Enzymol.* 18b, 51.
166. Krakow, G., Ludowieg, J., Mathes, J. H., Tosi, L., Udaka, S., and Vennesland, B. (1963). *Biochemistry* 2, 1009.
167. Bjorkhem, I., and Danielsson, H. (1970). *Eur. J. Biochem.* 12, 80.
168. Zimmerman, S. B., Little, J. W., Oshinsky, C. K., and Gellert, M. (1967). *Proc. Natl. Acad. Sci. U.S.A.* 57, 1841.
169. Ueda, K., and Yamamura, H. (1971). *Methods Enzymol.* 18b, 60.
170. Oppenheimer, N. J., Arnold, L. J., and Kaplan, N. O. (1971). *Proc. Natl. Acad. Sci. U.S.A.* 68, 3200.
171. Viola, R. E., Cook, P. F., and Cleland, W. W. (1979). *Anal. Biochem.* 96, 334.
172. Fisher, H. F., Coon, E. E., Vennesland, B., and Westheimer, F. H. (1953). *J. Biol. Chem.* 202, 687.
173. de Juan, E., and Taylor, K. B. (1976). *Biochemistry* 15, 2523.
174. San Pietro, A. (1959). *J. Biol. Chem.* 217, 579.
175. Arnold, L. J., You, K., Allison, W. S., and Kaplan, N. O. (1976). *Biochemistry* 15, 4844.
176. Fruedenthal, R. I., Kepler, J. A., and Cook, C. E. (1973). *Anal. Biochem.* 51, 632.
177. Oppenheimer, N. J., Arnold, L. J., and Kaplan, N. O. (1978). *Biochemistry* 17, 2613.
178. Christ, W., and Coper, H. (1969). *FEBS Lett.* 2, 267.
179. Anderson, B. M., and Dubler, R. E. (1980). *Arch. Biochem. Biophys.* 200, 583.
180. Lee, C. Y., and Everse, J. (1973). *Arch. Biochem. Biophys.* 157, 83.
181. Secrist, J. A., and Leonard, N. J. (1972). *J. Am. Chem. Soc.* 94, 1702.
182. Barrio, J. R., Tolman, G. L., Leonard, N. J., Spencer, R. D., and Weber, G. (1973). *Proc. Natl. Acad. Sci. U.S.A.* 70, 941.
183. Scott, T. G., Spencer, R. D., Leonard, N. J., and Weber, G. (1970). *J. Am. Chem. Soc.* 92, 687.
184. Luisi, P. L., Baici, A., Bonner, F. J., and Aboderin, A. A. (1975). *Biochemistry* 14, 362.
185. Wallenfels, K., and Diekmann, H. (1959). *Justus Liebig's Ann. Chem.* 621, 166.
186. Burton, K., and Wilson, T. H. (1953). *Biochem. J.* 54, 86.
187. Rodkey, F. L. (1955). *J. Biol. Chem.* 213, 777.
188. Colowick, S. P., van Eys, J., and Park, J. H. (1966). *In* "Comprehensive Biochemistry," (M. Florkin, and E. H. Stotz, eds.), Vol. 14, p. 1. Elsevier, Amsterdam.
189. Everse, J., and Kaplan, N. O. (1973). *Adv. Enzymol.* 37, 61.
190. Winer, A. D. (1958). *Acta Chem. Scand.* 12, 1695.
191. Pietruszko, R., Crawford, K., and Lester, D. (1973). *Arch. Biochem. Biophys.* 159, 50.
192. Welsh, K. M., Creighton, D. J., and Klinman, J. P. (1980). *Biochemistry* 19, 205.
193. Racker, E. (1950). *J. Biol. Chem.* 184, 313.
194. Holzer, H., and Soling, H. D. (1962). *Biochem. Z.* 336, 201.
195. Levy, H. R., and Daouk, G. H. (1979). *J. Biol. Chem.* 254, 4843.
196. Olive, C., Geroch, M. E., and Levy, H. R. (1971). *J. Biol. Chem.* 246, 2047.
197. Beisenherz, G., Boltze, H. J., Bucher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G. (1953). *Z. Naturforsch.* 8b, 555.
198. Olomucki, A., Thome-Beau, F., Biellmann, J. F., and Branlant, G. (1975). *Eur. J. Biochem.* 56, 109.
199. Baici, A., Luisi, P. L., Olomucki, A., Doublet, M. O., and Klincak, J. (1974). *Eur. J. Biochem.* 46, 59.
200. Anderson, B. M., Yuan, J. H., and Vercel
201. Zens, A. P., Bryson, T. A., Dunlap, R. B *Chem. Soc.* 98, 7559.
202. Reisbig, R. R., and Woody, R. W. (1978)
203. Sovago, I., and Martin, R. B. (1979). *FEI*
204. Gruber, B. A., and Leonard, N. J. (1975).
205. Gafni, A. (1977). *In* "Pyridine Nucleotid 237. de Gruyter, Berlin.
206. Bonner, F., Aboderin, A. A., Klincak, J., 211.
207. Baici, A., and Luisi, P. L. (1974). *Experi*
208. Schlessinger, J., and Levitzki, A. (1974).
209. Schlessinger, J., Levitzki, A., and Steinbe
210. Gafni, A. (1979). *Biochemistry* 18, 1540.
211. Stein, A. M., Kaplan, N. O., and Ciotti, I
212. Kaplan, N. O. (1967). *Methods Enzymol.*
213. Hatefi, Y., and Hanstein, W. G. (1973). *I*
214. Djavadi-Ohaniance, L., and Hatefi, Y. (19
215. Cohen, P. T., and Kaplan, N. O. (1970).
216. Klingenberg, M., and Slenczka, W. (1959
217. Louie, D. D., Kaplan, N. O., and Lean, J
218. Kaplan, N. O. (1972). *Harvey Lect.* 54, 1
219. Cohen, P. T., and Kaplan, N. O. (1970).
220. van den Broek, H. W. J., Santena, J. S., *Biochem.* 24, 31.
221. Moroff, G., Ochs, R. S., and Brandt, K.
222. Buckon, M. E., and Anderson, B. M. (19
223. Pekala, P. H., Yost, D. A., and Anderson
224. Skala, H., Vibert, M., Kahn, A., and Dre *nun.* 89, 988.
225. Wofsy, L., Metzger, H., and Singer, S. J.
226. Chen, S., and Guillory, R. J. (1980). *J. B*
227. Bayley, H., and Knowles, J. R. (1978). *B*
228. Bayley, H., and Knowles, J. R. (1978). *B*
229. Ong, E. G., Shaw, E., and Schoellmann,
230. Shaw, E., Mares-Guia, M., and Cohen, W
231. Kafatos, F. C., Law, J. H., and Tartakoff
232. Biellmann, J. F., Eid, P., Goulas, P. R.,
233. Biellmann, J. F., Branlant, G., Faucaud Nucleotide-Dependent Dehydrogenases" (i
234. Woenckhaus, C., Zolkobrocki, M., Bergh *354*, 60.
235. Woenckhaus, C., Jeck, R., Dietz, G., and
236. Jornvall, H., Woenckhaus, C., Schartle, E
237. Eklund, H., Nordstrom, B., Zeppezauer, I berg, B. O., Tapia, O., Branden, C. I., a
238. Jornvall, H. (1977). *In* "Pyridine Nucleoti 62. de Gruyter, Berlin.
239. Jornvall, H., Eklund, H., and Branden, C
240. Li, T. K., and Vallee, B. L. (1963). *Biod*

200. Anderson, B. M., Yuan, J. H., and Vercelloni, S. V. (1975). *Mol. Cell. Biochem.* **8**, 89.
201. Zens, A. P., Bryson, T. A., Dunlap, R. B., Fisher, R. R., and Ellis, P. D. (1976). *J. Am. Chem. Soc.* **98**, 7559.
202. Reisbig, R. R., and Woody, R. W. (1978). *Biochemistry* **17**, 1974.
203. Sovago, I., and Martin, R. B. (1979). *FEBS Lett.* **106**, 132.
204. Gruber, B. A., and Leonard, N. J. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3966.
205. Gafni, A. (1977). *In* "Pyridine Nucleotide-Dependent Dehydrogenases" (H. Sund, ed.), p. 237. de Gruyter, Berlin.
206. Bonner, F., Aboderin, A. A., Klincak, J., and Luisi, P. L. (1973). *Z. Physiol. Chem.* **354**, 211.
207. Baici, A., and Luisi, P. L. (1974). *Experientia* **30**, 685.
208. Schlessinger, J., and Levitzki, A. (1974). *J. Mol. Biol.* **82**, 547.
209. Schlessinger, J., Levitzki, A., and Steinberg, I. Z. (1975). *J. Mol. Biol.* **91**, 523.
210. Gafni, A. (1979). *Biochemistry* **18**, 1540.
211. Stein, A. M., Kaplan, N. O., and Ciotti, M. M. (1959). *J. Biol. Chem.* **234**, 979.
212. Kaplan, N. O. (1967). *Methods Enzymol.* **10**, 317.
213. Hatefi, Y., and Hanstein, W. G. (1973). *Biochemistry* **12**, 3515.
214. Djavadi-Ohaniance, L., and Hatefi, Y. (1975). *J. Biol. Chem.* **250**, 9397.
215. Cohen, P. T., and Kaplan, N. O. (1970). *J. Biol. Chem.* **245**, 4666.
216. Klingenberg, M., and Slenczka, W. (1959). *Biochem. Z.* **331**, 486.
217. Louie, D. D., Kaplan, N. O., and Lean, J. D. (1972). *J. Mol. Biol.* **70**, 651.
218. Kaplan, N. O. (1972). *Harvey Lect.* **54**, 105.
219. Cohen, P. T., and Kaplan, N. O. (1970). *J. Biol. Chem.* **245**, 2825.
220. van den Broek, H. W. J., Santena, J. S., Wassink, J. H., and Veeger, C. (1971). *Eur. J. Biochem.* **24**, 31.
221. Moroff, G., Ochs, R. S., and Brandt, K. G. (1976). *Arch. Biochem. Biophys.* **173**, 42.
222. Buckon, M. E., and Anderson, B. M. (1980). *Arch. Biochem. Biophys.* **202**, 396.
223. Pekala, P. H., Yost, D. A., and Anderson, B. M. (1980). *Mol. Cell. Biochem.* **31**, 49.
224. Skala, H., Vibert, M., Kahn, A., and Dreyfus, J. C. (1979). *Biochem. Biophys. Res. Commun.* **89**, 988.
225. Wofsy, L., Metzger, H., and Singer, S. J. (1962). *Biochemistry* **1**, 1013.
226. Chen, S., and Guillory, R. J. (1980). *J. Biol. Chem.* **255**, 2445.
227. Bayley, H., and Knowles, J. R. (1978). *Biochemistry* **17**, 2414.
228. Bayley, H., and Knowles, J. R. (1978). *Biochemistry* **17**, 2420.
229. Ong, E. G., Shaw, E., and Schoellmann, G. (1964). *J. Am. Chem. Soc.* **86**, 1271.
230. Shaw, E., Mares-Guia, M., and Cohen, W. (1965). *Biochemistry* **4**, 2219.
231. Kafatos, F. C., Law, J. H., and Tartakoff, A. M. (1967). *J. Biol. Chem.* **242**, 1488.
232. Biellmann, J. F., Eid, P., Goulas, P. R., and Hirth, C. (1978). *Biochimie* **60**, 417.
233. Biellmann, J. F., Branlant, G., Faucaud, B. Y., and Wallen, L. (1977). *In* "Pyridine Nucleotide-Dependent Dehydrogenases" (H. Sund, ed.), p. 249. de Gruyter, Berlin.
234. Woenckhaus, C., Zoltobrocki, M., Berghauser, J., and Jeck, R. (1973). *Z. Physiol. Chem.* **354**, 60.
235. Woenckhaus, C., Jeck, R., Dietz, G., and Jentsch, G. (1973). *FEBS Lett.* **34**, 175.
236. Jornvall, H., Woenckhaus, C., Schattle, E., and Jeck, R. (1975). *FEBS Lett.* **54**, 297.
237. Eklund, H., Nordstrom, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., Soderberg, B. O., Tapia, O., Branden, C. I., and Akeson, A. (1976). *J. Mol. Biol.* **102**, 27.
238. Jornvall, H. (1977). *In* "Pyridine Nucleotide-Dependent Dehydrogenases" (H. Sund, ed.), p. 62. de Gruyter, Berlin.
239. Jornvall, H., Eklund, H., and Branden, C. I. (1978). *J. Biol. Chem.* **253**, 8414.
240. Li, T. K., and Vallee, B. L. (1963). *Biochem. Biophys. Res. Commun.* **12**, 44.

241. Harris, I. (1964). *Nature (London)* **203**, 30.
242. Li, T. K., and Vallee, B. L. (1964). *Biochemistry* **3**, 869.
243. Eklund, H., Nordstrom, B., Zeppesauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., and Branden, C. I. (1974). *FEBS Lett.* **44**, 200.
244. Woenckhaus, C., Jeck, R., and Jornvall, H. (1979). *Eur. J. Biochem.* **93**, 65.
245. Biellmann, J. F., Goulas, P. R., and Dallocchio, F. (1978). *Eur. J. Biochem.* **88**, 433.
246. Biellmann, J. F., Eid, P., and Hirth, C. (1980). *Eur. J. Biochem.* **104**, 65.
247. Biellmann, J. F., Goulas, P. R., Nicolas, J. C., Descomps, B., and Crastes de Paulet, A. (1979). *Eur. J. Biochem.* **99**, 81.
248. Jeck, R., Woenckhaus, C., Harris, J. I., and Runswick, M. J. (1979). *Eur. J. Biochem.* **93**, 57.
249. Dietz, G., Woenckhaus, C., Jaenicke, R., and Schuster, I. (1977). *Z. Naturforsch.* **32c**, 85.
250. Jaenicke, R., Krebs, H., Rudolph, R., and Woenckhaus, C. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1966.
251. Anderson, B. M., Kohler, S. T., and Anderson, C. D. (1978). *Arch. Biochem. Biophys.* **188**, 214.
252. Hess, G. P., and Rupley, J. A. (1971). *Annu. Rev. Biochem.* **40**, 1036.
253. Amy, N. K., Garrett, R. H., and Anderson, B. M. (1977). *Biochim. Biophys. Acta* **480**, 83.
254. Dubler, R. E. (1980). Ph.D. Thesis, Virginia Polytechnic Inst. and State Univ., Blacksburg.
255. Kaplan, N. O., Goldin, A., Humphreys, S. R., Ciotti, M. M., and Venditti, J. M. (1954). *Science (Washington, D.C.)* **120**, 437.
256. Kaplan, N. O. (1976). *In* "Cancer Enzymology" (J. Schulz, and F. Ahmod, eds.), Vol. 12, p. 201. Academic Press, New York.
257. Brunnemann, A., Coper, H., and Herken, H. (1962). *Arch. Exp. Pathol. Pharmakol.* **244**, 223.
258. Brunnemann, A., Coper, H., and Herken, H. (1963). *Arch. Exp. Pathol. Pharmakol.* **245**, 541.
259. Herken, H., and Neuhoff, V. (1964). *Arch. Exp. Pathol. Pharmakol.* **247**, 187.
260. Shen, W. C., Greene, K. M., and Van Vunakis, H. (1977). *Biochem. Pharmacol.* **26**, 1841.
261. DiAugustine, R. P. (1976). *Mol. Pharmacol.* **12**, 291.
262. McColl, J. D., Rice, W. B., and Adamkiewicz, V. W. (1957). *Can. J. Biochem.* **35**, 795.
263. Ross, W. C. J. (1967). *Biochem. Pharmacol.* **16**, 675.
264. Shapiro, D. M., Dietrich, L. S., and Shils, M. E. (1957). *Cancer Res.* **17**, 600.
265. Herter, F. P., Wiessman, S. G., Thompson, H. G., Jr., Hyman, G., and Martin, D. S. (1961). *Cancer Res.* **21**, 31.
266. Perlia, C. P., Kofman, S., Sky-Peck, H., and Taylor, S. G., III. (1961). *Cancer* **14**, 644.
267. Cowan, D. H., and Alison, R. E. (1970). *Cancer Chemother. Rep.* **54**, 175.
268. Zackheim, H. S. (1975). *Arch. Dermatol.* **111**, 880.
269. Zackheim, H. S. (1978). *Arch. Dermatol.* **114**, 1632.
270. Brunnemann, A., Coper, H., and Neubert, D. (1964). *Arch. Exp. Pathol. Pharmakol.* **246**, 437.
271. Coper, H., and Neubert, D. (1964). *Biochim. Biophys. Acta* **89**, 23.
272. Herken, H., Lange, K., Kolbe, H., and Keller, K. (1974). *In* "Central Nervous System" (E. Genazzani and H. Herken, eds.), p. 41. Springer-Verlag, Berlin and New York.
273. Lange, K., Kolbe, H., Keller, K., and Herken, H. (1970). *Z. Physiol. Chem.* **351**, 1241.
274. Keller, K., Kolbe, H., Lange, K., and Herken, H. (1972). *Z. Physiol. Chem.* **353**, 1389.
275. Herken, H., and Lange, K. (1969). *Arch. Exp. Pathol. Pharmakol.* **263**, 496.
276. Halprin, K. M., and Ohkawara, A. (1966). *J. Invest. Dermatol.* **46**, 51.
277. Weber, G., and Ganzer, U. (1966). *Arch. Klin. Exp. Dermatol.* **226**, 222.
278. Wolf, A., and Cowan, D. (1959). *Bull. N.Y. Acad. Med.* **35**, 814.

279. Turbow, M. M., Clark, W. H., and Dipa
280. Hicks, S. P. (1955). *Amer. J. Pathol.* 31.
281. Coggeshall, R. E., and MacLean, P. D.
282. Vessell, E. S., and Bearn, A. G. (1958).
283. Wilkinson, J. H. (1970). *In* "Isozymes,"
284. Wiene, R. J., and Van Maercke, Y. (196
285. Wilkinson, J. H. (1970). *Clin. Chem.* **16**,
286. Bishop, M. J., Everse, J., and Kaplan, N.
287. Fujisawa, K., Kimura, A., Minato, S., Ta
Acta **69**, 251.
288. Tamaoki, H., Mizushima, H., Takei, S.
Kenkyusho Nempo **25**, 85.
289. Sund, H., and Theorell, H. (1963). *In*
Myrback, eds.), 2nd ed., vol. 7, p. 29. A
290. Kaplan, N. O., Ciotti, M. M., Hamn
(*Washington, D.C.*) **131**, 392.

279. Turbow, M. M., Clark, W. H., and Dipaolo, J. A. (1971). *Teratology* 4, 427.
280. Hicks, S. P. (1955). *Amer. J. Pathol.* 31, 189.
281. Coggeshall, R. E., and MacLean, P. D. (1958). *Proc. Soc. Exp. Biol. Med.* 98, 687.
282. Vessell, E. S., and Bearn, A. G. (1958). *J. Clin. Invest.* 37, 672.
283. Wilkinson, J. H. (1970). *In* "Isozymes," p. 43. Lippincott, Philadelphia, Pennsylvania.
284. Wiene, R. J., and Van Maercke, Y. (1961). *Ann. N.Y. Acad. Sci.* 94, 898.
285. Wilkinson, J. H. (1970). *Clin. Chem.* 16, 733.
286. Bishop, M. J., Everse, J., and Kaplan, N. O. (1972). *Proc. Natl. Acad. Sci. U.S.A.* 69, 1761.
287. Fujisawa, K., Kimura, A., Minato, S., Tamaoki, H., and Mizushima, H. (1976). *Clin. Chim. Acta* 69, 251.
288. Tamaoki, H., Mizushima, H., Takei, S., Minato, S., and Nakanishi, K. (1973). *Sankyo Kenkyusho Nempo* 25, 85.
289. Sund, H., and Theorell, H. (1963). *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrback, eds.), 2nd ed., vol. 7, p. 29. Academic Press, New York.
290. Kaplan, N. O., Ciotti, M. M., Hamolsky, M., and Bieber, R. E. (1960). *Science* (*Washington, D.C.*) 131, 392.

[11] Transition State and Multisubstrate Analog Inhibitors

By ANNA RADZICKA and RICHARD WOLFENDEN

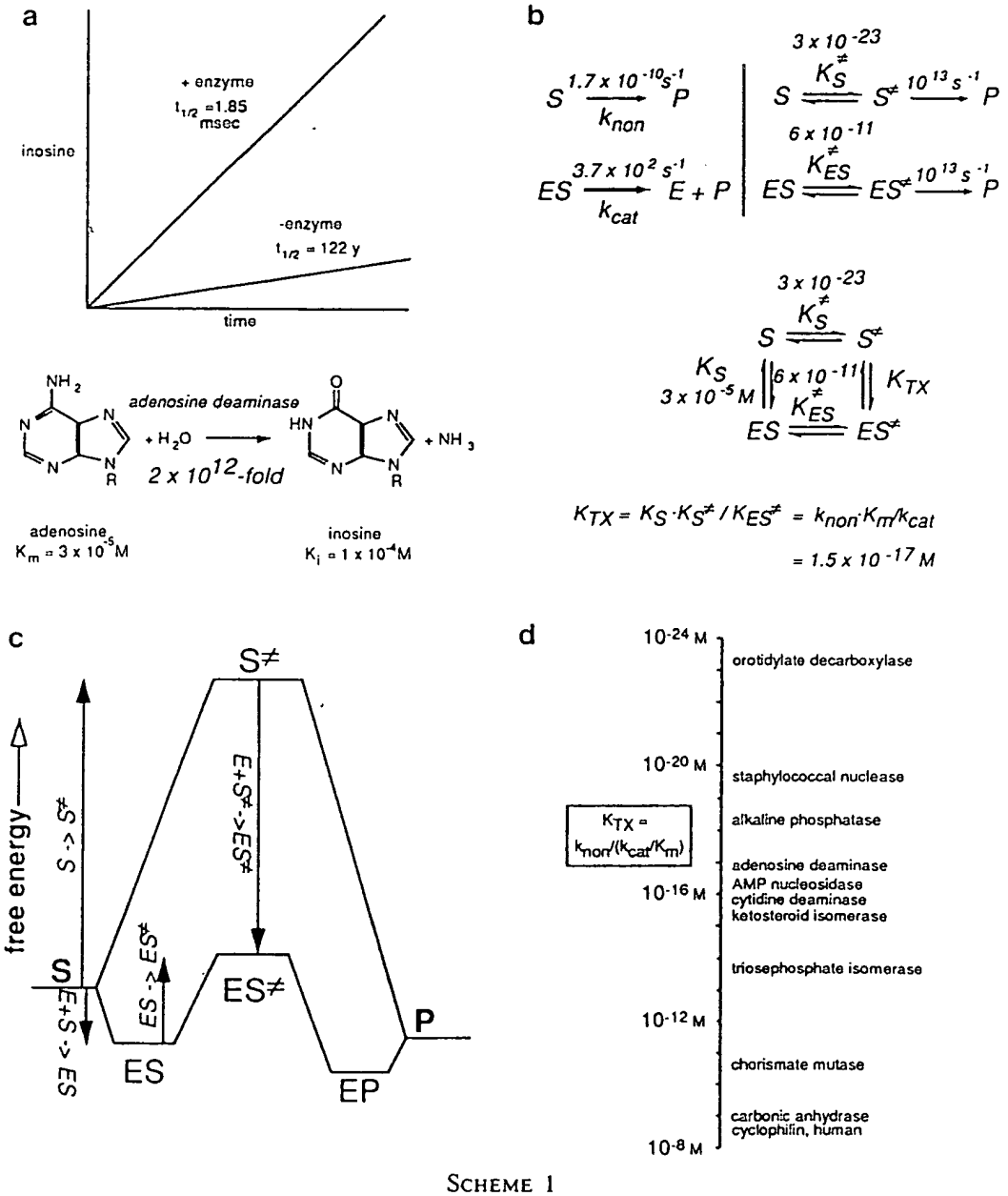
Transition State Stabilization by Enzymes

To lower the energy barrier that limits the rate of a reaction, a catalyst must bind the altered substrate in the transition state ($S^{\ddagger}$) more tightly than it binds the substrate in the ground state (S). In the moment, lasting perhaps 1 msec, during which the catalytic event occurs, binding is enhanced by a factor that equals or surpasses the factor by which the catalyst enhances the rate of the reaction.[1]

Scheme 1 illustrates this principle by comparing the rate of deamination of adenosine in the presence and absence of calf intestinal adenosine deaminase (Scheme 1a). The central postulate of transition state theory is that in the ground state the substrate S (in the nonenzymatic reaction), or the enzyme–substrate complex ES (in the enzyme-catalyzed reaction), exists in a state of equilibrium with a transition state ($S^{\ddagger}$ or $ES^{\ddagger}$, respectively) situated at the top of a potential energy barrier, from which its chances of going forward to products, or backward to substrates, are equal (Schemes 1b and 1c).

The rate of decomposition of any transition state to products is equal to kT/h, a universal rate constant composed of Planck's constant k, Boltzmann's constant h, and the absolute temperature T; and it has a value of approximately 0.62×10^{13} sec^{-1} at room temperature. At any given temperature, rates of reactions differ according to the difference between their equilibrium constants for reaching the transition state. That equilibrium constant is always unfavorable, even for the fastest enzyme reactions. Thus, for carbonic anhydrase, with a turnover number of roughly 10^6 sec^{-1},[2] the value of $K_{ES^{\ddagger}}$ is about 10^{-7}. For calf intestinal adenosine deaminase, a more conventional enzyme with a turnover number of 375 sec^{-1}, the value of $K_{ES^{\ddagger}}$ is approximately 6×10^{-11}. At neutral pH in water, adenosine is deaminated very slowly ($k_{non} = 1.8 \times 10^{-10}$ sec^{-1}; $t_{1/2} = 122$ years),[3] and the value of $K_{S^{\ddagger}}$ is approximately 3×10^{-23}. The ratio of $K_{ES^{\ddagger}}$ to $K_{S^{\ddagger}}$ matches the rate enhancement of 2×10^{12}-fold. Scheme 1b shows that K_S is related to K_{TX} by this same ratio, where K_S is the dissociation constant of the enzyme–substrate com-

[1] R. Wolfenden, *Nature (London)* **223**, 704 (1969).
[2] H. Steiner, B. H. Johnson, and S. Lindskog, *Eur. J. Biochem.* **59**, 253 (1975).
[3] L. Frick, J. P. Mac Neela, and R. Wolfenden, *Bioorg. Chem.* **15**, 100 (1987).



SCHEME 1

plex and K_{TX} is the dissociation constant of the enzyme–substrate complex in the transition state. Evidently the substrate, initially bound with a dissociation constant of roughly 3×10^{-5} M, is bound with a dissociation constant of approximately 1.5×10^{-17} M in the transition state.

In the simple case described in Scheme 1, K_{TX} (expressed in moles/

liter) is equivalent to k_{non} (the first-order rate constant for the nonenzymatic reaction, usually expressed as sec^{-1}) divided by k_{cat}/K_m (the second-order rate constant for the enzyme reaction, usually expressed as $sec^{-1} M^{-1}$).

Second-order rate constants for enzyme reactions typically fall in the range between 10^5 and 10^7 $sec^{-1} M^{-1}$. Rate constants for the corresponding nonenzymatic reactions are distributed over a much wider range, with half-times that may be measured in tens of seconds or millions of years. Scheme 1d shows approximate values of K_{TX} for several enzymes, estimated from such measurements. The resulting affinities of $S^{\ddagger}$ are seen to be high. In most cases, the observed rate enhancement probably provides a conservative estimate of the levels of binding affinity that are achieved in the transition state. As has been shown elsewhere,[4] transition state affinity may be underestimated, from simple comparison of rates, if (1) the enzymatic and nonenzymatic reactions differ mechanistically in some fundamental respect, so that their transition states are unrelated in structure; or (2) the chemical mechanisms are the same, but the rate-limiting transition state is reached at a different point on the reaction coordinate in the enzymatic and nonenzymatic reactions. In both cases, simple comparison of rates results in underestimation of the ability of the enzyme to stabilize the transition state for the step that limits the rate of the nonenzymatic reaction.

This view of catalysis, focusing attention on a concrete structure rather than a process, implies that the catalytic power of enzymes lies in their extremely high affinity for unstable intermediates in substrate transformation, as opposed to the substrate in the ground state. The rate of the nonenzymatic reaction can be enhanced only if this difference in binding affinities exists. This principle also furnishes a practical basis for designing powerful enzyme antagonists, in the form of stable analogs of $S^{\ddagger}$. These inhibitors, usually termed "transition state analogs," can be designed to test alternative mechanisms by which the enzyme might act; that is, strong binding should be observed only for those inhibitors that resemble activated forms of the substrate that arise along the pathway of the enzyme reaction. Such inhibitors can be used to probe the source of the binding discrimination of the enzyme between the substrate in the ground state and that in the transition state, which lies at the heart of the catalytic process. Thus, exact structural observations on enzyme–inhibitor complexes should make it possible to identify the origins of the affinity of the enzyme for the inhibitor and, by inference, the interactions that stabilize

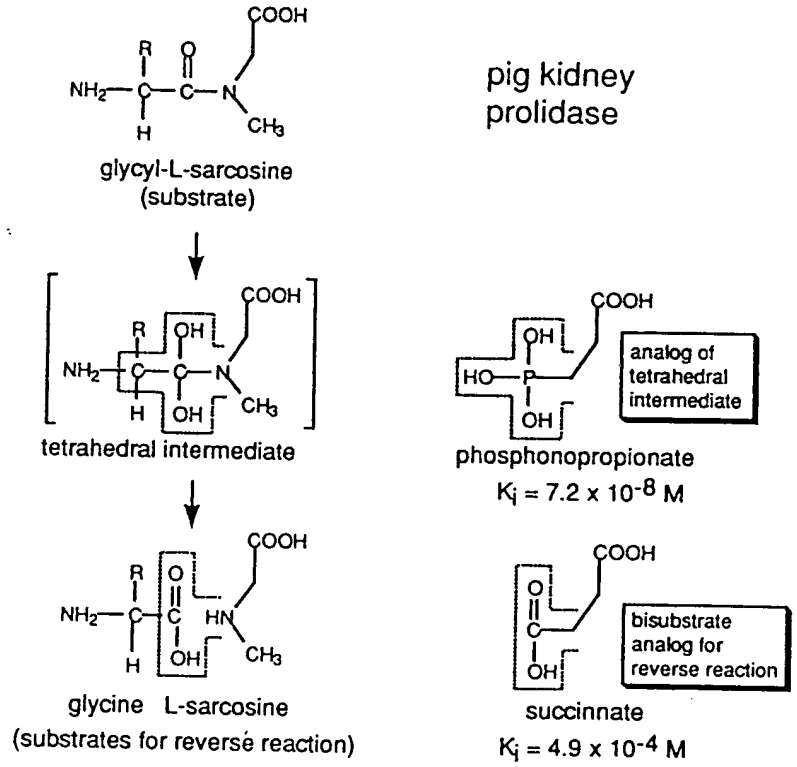
[4] For a review, see R. Wolfenden, *Annu. Rev. Biophys.* 5, 271 (1976).

the actual transition state for the reaction, involving those amino acid residues of the enzyme that are directly involved in catalysis.

The transition state, by definition the highest point on the energy profile of a reaction, involves bond angles, bond distances, and electron distributions that can never be imitated precisely in any stable analog inhibitor. In addition, some compounds designed on this principle resemble reaction intermediates whose structures and energies may approach, but inevitably fall short of, that of the altered substrate in the transition state itself. For these reasons, the term "transition state analog" describes an ideal that will never be fully attained. Nevertheless, $S^{\ddagger}$ represents an ideal that is worth imitating, because a compound that shares even a few of the structural features that distinguish $S^{\ddagger}$ from S should be a very strong inhibitor, many orders of magnitude more strongly bound than the substrate or product. Potential transition state analog inhibitors have now been prepared against enzymes catalyzing reactions of every class (see Table I at the end of this chapter), and some show very high affinities. For example, 1,6-dihydroinosine (or nebularine 1,6-hydrate, described below) is bound by intestinal adenosine deaminase 3×10^8-fold more tightly than product inosine[5]; 3,4-dihydrouridine is bound by bacterial cytidine deaminase 2×10^9-fold more tightly than product uridine.[6]

A special kind of activation involves gathering of two or more substrates from dilute solution at the active site and their binding in an orientation appropriate for reaction. A multisubstrate analog inhibitor that incorporates binding determinants of two or more substrates within the same molecule may express a large entropic advantage in binding, as compared with the binding properties of analogs of the two substrates measured separately.[7] In principle[8] and in practice,[9] these effects can enhance reaction rates and inhibitor binding affinities by factors as large as 10^8 or more. Accordingly, given only that a compound is an exceptionally powerful inhibitor of a multisubstrate reaction, it may not be easy to decide whether its potency is due to some resemblance to the combined substrates, or to a chemically activated intermediate in which bonds are being made and broken. In some cases, such as that shown in Scheme 2, one inhibitor, tentatively identified as a multisubstrate analog inhibitor, is greatly sur-

[5] W. Jones, L. C. Kurz, and R. Wolfenden, *Biochemistry* **28**, 1242 (1989).
[6] L. Frick, C. Yang, V. E. Marquez, and R. Wolfenden, *Biochemistry* **28**, 9423 (1989).
[7] R. Wolfenden, *Acc. Chem. Res.* **5**, 10 (1972).
[8] M. I. Page and W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1678 (1971).
[9] W. M. Kati, S. A. Acheson, and R. Wolfenden, *Biochemistry* **31**, 7356 (1992).



SCHEME 2

passed in potency by another inhibitor containing a bond arrangement that resembles a hydrated intermediate in peptide cleavage.[10] The first of these compounds might be considered a multisubstrate analog, whereas the second has some of the structural features expected of a transition state.

Designing Transition State Analogs

Transition state and multisubstrate inhibitors have been prepared against enzymes catalyzing reactions of every class (see Table I). Each of the various devices by which enzymes are able to enhance the rates of reactions that they catalyze, including catalysis by approximation, general acid–base catalysis, catalysis by desolvation, nucleophilic catalysis, and catalysis by distortion, now appears to have been exploited in several inhibitors (for a review, see Wolfenden and Frick[11]). In what follows, we

[10] A. Radzicka and R. Wolfenden, *Biochemistry* 30, 4160 (1991).
[11] R. Wolfenden and L. Frick, *in* "Enzyme Mechanisms" (M. I. Page and A. Williams, eds.), p. 9. Royal Society of Chemistry, London, 1987.

consider a few such inhibitors that have been grouped into several classes that emphasize their charge, reactivity with nucleophiles, hydrophobicity, or multisubstrate character.

Analogs of Anionic Intermediates

Isotope exchange studies have shown that many enzymes abstract protons from substrates, generating carbanionic or oxyanionic intermediates that undergo subsequent addition or rearrangement reactions. Inhibitory compounds resembling these species can be prepared by incorporation of stable oxyanionic substituents such as carboxylate groups.

Triose-phosphate isomerase, for example, was found to be strongly inhibited by 2-phosphoglycolate, analogous in structure to a suspected ene-diolate intermediate in the enzyme reaction. Judging from the effect of pH on K_i, the inhibitor appeared to be bound as a dianion.[12] However, ^{13}C and ^{31}P nuclear magnetic resonance experiments showed that the inhibitor was bound as the trianion, requiring that the enzyme have taken up a proton as binding occurred.[13] It was inferred that the EI complex resembled a species on the reaction pathway in which a basic group on the enzyme, Glu-165, had abstracted a proton from the substrate to form an ene-diolate intermediate, and that interpretation has been confirmed by X-ray crystallography of the EI complex.[14] The true affinity of the enzyme for the inhibitor is therefore several orders of magnitude higher than the K_i value had suggested. A comparable approach has been used to examine the complex formed between carboxypeptidase A and the multisubstrate analog inhibitor L-benzylsuccinate. From the dependence of K_i on pH, the inhibitor appeared to be bound as one of two possible monoanionic species involving the two carboxylic acid groups of the inhibitor.[15] When these groups were substituted with ^{13}C, resonances of the bound inhibitor showed that it was bound instead as the dianionic species, with concomitant release of a hydroxide ion from the protein.[16]

Barbituric acid ribonucleoside 5′-phosphate is an excellent inhibitor of orotidylate decarboxylase from yeast, presumably because one of its canonical forms resembles a zwitterionic intermediate formed during decarboxylation.[17] The resonance of ^{13}C at C-5, as well as the UV difference

[12] R. Wolfenden, *Biochemistry* **9**, 3404 (1970).
[13] I. D. Campbell, R. B. Jones, P. A. Keiner, E. Richards, S. G. Waley, and R. Wolfenden, *Biochem. Biophys. Res. Commun.* **83**, 347 (1978).
[14] E. Lolis and G. A. Petsko, *Biochemistry* **29**, 6619 (1990).
[15] L. D. Byers and R. Wolfenden, *Biochemistry* **12**, 2070 (1973).
[16] A. R. Palmer, P. D. Ellis, and R. Wolfenden, *Biochemistry* **21**, 5056 (1982).
[17] H. L. Levine, R. S. Brody, and F. H. Westheimer, *Biochemistry* **19**, 4993 (1980).

spectrum of the bound inhibitor, shows that it is bound in zwitterionic form, not as a covalent 5,6-adduct that would have been expected if the enzyme had acted by an alternative mechanism involving addition and elimination of an enzyme nucleophile.[18]

Analogs of Cationic Intermediates

Carbonium ion intermediates are generated during acid-catalyzed hydrolysis of glycosides, and also during the action of most glycosidases. In the enzyme reactions, a covalent glycosyl–enzyme intermediate may also be formed, but carbonium ions intervene during its formation and breakdown. Presumably for this reason, several glycosidases are strongly inhibited by 1-amino sugars whose protonated forms may form ion pairs with carboxylate groups at the active site that normally serve as a source of protons to the leaving group.[17]

Sterol methyltransferases are believed to involve nucleophilic attack by the sterol on the methyl function of *S*-adenosylmethionine. The methylated intermediate contains a positive charge on an adjacent tertiary carbon atom, and when this atom is replaced by nitrogen potent inhibition results.[2] Squalene synthetase is believed to generate a carbonium ion adjacent to a cyclopropane ring, by elimination of a pyrophosphoryl group. Rearrangement of the intermediate, followed by hydride transfer, leads to squalene. An analogous azasterol serves as a strong inhibitor.[12]

Electrophilic Analogs

Many hydrolases and transferases act by a double displacement mechanism, in which an enzyme nucleophile displaces part of the substrate to form a covalently bound intermediate that undergoes hydrolysis or transfer in a second step. Enzymes involved in carboxyl (or phosphoryl) transfer reactions are often susceptible to inhibition by analogs that undergo the first stage of reaction, typically forming a tetrahedral (or trigonal bipyramidal) intermediate but stop at that stage because they lack an appropriate leaving group. Thus, aldehyde analogs of peptides and amides, in which a hydrogen atom replaces the normal leaving group, are powerful inhibitors of proteases with cysteine[19] or serine[20] nucleophiles at the active site, forming hemiacetals whose stability reflects (1) the ability of the enzyme to stabilize tetrahedral intermediates in substitution and (2) the unusually favorable equilibrium constant for addition of nucleophiles to aldehydes.

[18] S. A. Acheson, J. B. Bell, M. E. Jones, and R. Wolfenden, *Biochemistry* **29**, 3198 (1990).
[19] J. O. Westerik and R. Wolfenden, *J. Biol. Chem.* **247**, 8195 (1972).
[20] R. C. Thompson, *J. Biol. Chem.* **12**, 47 (1973).

When such an enzyme is also specific for the leaving group, an additional advantage can be gained by using a ketone in which one substituent represents the acyl group and the other substituent represents the leaving group. Equilibria of addition of nucleophiles to ketones are much less favorable than for addition to aldehydes, but this effect can be offset by incorporating fluoro groups to promote electrophilic character. In this way, inhibitors of remarkable potency were developed for acetylcholinesterase and several proteases.[21]

Instead of mediating a double displacement reaction, some hydrolases catalyze direct transfer to the acceptor water. Aldehydes and ketones can also inhibit reactions of these kinds, not by addition of an enzyme nucleophile, but by addition of water itself to form a *gem*-diol that resembles a tetrahedral intermediate in direct water attack on the peptide bond. Demonstrated first for pepsin inhibitors containing the unnatural amino acid statone,[22] this mode of binding has also been observed in complexes of fluoroketones with carboxypeptidase A.[23]

Enzymes of the latter type are extremely strongly inhibited by a special class of compounds in which a $-P(=O)O^-—NH_2-$ group replaces the peptide bond, allowing four substituents (including an oxygen anion) to interact with the active site in a manner resembling an oxyanionic intermediate in peptide hydrolysis,[24,25] and this mode of binding has been confirmed by X-ray crystallography.[26] This combination of features confers on these compounds very high binding affinities that may have been surpassed only by the complex formed between methionine sulfoximine phosphate and glutamine synthetase (glutamate–ammonia ligase).[27]

Analogs of Nonpolar Intermediates

Ethanol enhances the rate of decarboxylation of pyruvate by a factor of 10^4–10^5, approaching the value observed for pyruvate dehydrogenase catalyzing the same reaction.[28] This increase in the nonenzymatic rate was ascribed to the greater stability of the neutral resonance of the reactive ylide intermediate in the organic solvent, as compared with the charged

[21] M. H. Gelb, J. P. Svaren, and R. H. Abeles, *Biochemistry* **24,** 1813 (1985).
[22] D. H. Rich, A. S. Boparai, and M. S. Bernatowitz, *Biochem. Biophys. Res. Commun.* **104,** 3535 (1982).
[23] D. W. Christianson and W. N. Lipscomb, *J. Am. Chem. Soc.* **108,** 4998 (1986).
[24] P. A. Bartlett and C. K. Marlowe, *Biochemistry* **26,** 8553 (1987).
[25] A. P. Kaplan and P. A. Bartlett, *Biochemistry* **30,** 8165 (1991).
[26] B. W. Matthews, *Acc. Chem. Res.* **21,** 333 (1988).
[27] M. R. Maurizi and A. Ginsburg, *J. Biol. Chem.* **257,** 4271 (1982).
[28] J. Crosby and G. E. Lienhard, *J. Am. Chem. Soc.* **92,** 5707 (1970).

starting materials, and it was suggested that much of the transition state stabilization by pyruvate dehydrogenase was due to the hydrophobic nature of the active site. Keto or thioketo substitution at C-2 of the thiamine ring resulted in compounds with sp^3 hybridization at C-2, causing the ring nitrogen atom to lose its positive charge. These compounds proved to be extremely effective inhibitors or pyruvate dehydrogenase,[29] as did reduced TPP.[30]

Similarly, methyl transfer reactions involving positively charged *S*-adenosylmethionine probably involve charge dispersal in the transition state. Several nonpolar inhibitors of polyamine biosynthesis, synthesized by alkylation of thioamines with 5′-deoxy-5′-chloroadenosine, are considered to owe their effectiveness to hydrophobicity.[31]

Multisubstrate Analogs

Many enzymes play the role of a marriage broker, binding two or more substrates in a spatial relationship that is conducive to reaction. Multisubstrate analogs are single molecules that imitate the binding determinants in such a complex but save the enzyme the trouble of gathering the substrates from dilute solution. The first multisubstrate analog inhibitor ever prepared appears to have been pyridoxylalanine, a strong inhibitor of pyridoxamine–pyruvate transaminase.[32] Other early examples include L-benzylsuccinate, an inhibitor of carboxypeptidase A[33]; Ap_5A, a strong inhibitor of adenylate kinase[34]; and an inhibitor of ether lipid biosynthesis that incorporates the elements of both an attacking and a leaving group.[35] The number of such inhibitors is now very extensive, and, as discussed above, some may represent chemically activated species. One such analog, phosphorylated methionine sulfoximine, an inhibitor of glutamine synthetase,[36] appears to be bound with a K_d value in the range of 10^{-20} *M*.[27]

Practical Uses of Transition State Analogs

Transition state and multisubstrate analog inhibitors include a number of enzyme antagonists of practical importance. From a medicinal stand-

[29] J. A. Gutowski and G. E. Lienhard, *J. Biol. Chem.* **251,** 2863 (1976).
[30] P. N. Lowe, F. J. Leeper, and R. N. Perham, *Biochemistry* **22,** 150 (1983).
[31] K. C. Tang, A. E. Pegg, and J. K. Coward, *Biochem. Biophys. Res. Commun.* **96,** 1371 (1980).
[32] W. B. Dempsey and E. E. Snell, *Biochemistry* **2,** 1414 (1963).
[33] L. D. Byers and R. Wolfenden, *J. Biol. Chem.* **247,** 606 (1972).
[34] G. E. Lienhard and I. I. Secemski, *J. Biol. Chem.* **248,** 1121 (1973).
[35] S. Hixson and R. Wolfenden, *Biochem. Biophys. Res. Commun.* **101,** 1064 (1981).
[36] W. B. Rowe, R. A. Ronzio, and A. Meister, *Biochemistry* **8,** 2674 (1969).

point, these inhibitors represent attractive targets for drug design because the transition state tends to be uniquely characteristic of one kind of enzyme reaction, whereas substrates are usually shared by two or more enzymes. Most successful of these, from a clinical standpoint, have been antihypertensive inhibitors of the angiotensin-converting enzyme, namely, captopril[37] and enalapril,[38] whose design was based on benzylsuccinate, an early multisubstrate analog inhibitor of carboxypeptidase A.[33] A second major use of transition state analogs is as haptens in the production of catalytic antibodies, discussion of which is beyond the scope of this chapter (for reviews, see Lerner and Benkovic[39] and Shokat *et al.*[40]). Finally, transition state analogs constitute promising ligands for affinity chromatography and, more interestingly, as eluants from conventional substrate affinity columns. By using progressively increasing low concentrations of a transition state analog, enzymes can be eluted according to the molecular turnover numbers.[41]

Characterizing Transition State Analog Complexes

The affinities of transition state analogs for enzymes can be measured by methods conventionally used to characterize simple reversible inhibitors; however, when binding is extremely strong, the concentration of inhibitor present in kinetic experiments is so low that correction must be made for mutual depletion of the free enzyme and free inhibitor,[42] and for the time required for enzyme–inhibitor complexes to come to equilibrium. In cases of very tight binding, the best approach is to measure the "on" rate by determining the second-order rate constant for the onset of inhibition and the "off" rate by measuring the first-order rate constant for release of radiolabeled inhibitor in the presence of a large excess of unlabeled inhibitor. The quotient can be used to determine K_d values in the femtomolar range, which are inaccessible by other methods. Details of this method are given in papers describing applications to determining

[37] D. W. Cushman, H. S. Cheung, E. F. Sabo, and M. A. Ondetti, *Biochemistry* **16**, 5484 (1977).
[38] A. A. Patchett, E. Harris, E. W. Tristam, M. J. Wyvratt, M. T. Wu, D. Taub, E. R. Peterson, T. J. Ikeler, and J. ten Broeke, *Nature (London)* **288**, 280 (1980).
[39] R. A. Lerner and S. J. Benkovic, *Chemtracts—Org. Chem.* **3**, 1 (1990).
[40] K. M. Shokat, M. K. Ko, T. S. Scanlan, L. Kochersperger, S. Yonkovich, S. Thraisivongs, and P. G. Schultz, *Angew. Chem., Int. Ed. Engl.* **29**, 1296 (1990).
[41] L. Andersson and R. Wolfenden, *J. Biol. Chem.* **255**, 11106 (1980).
[42] J. L. Webb, *in* "Enzyme and Metabolic Inhibitors" Vol. 1, p. 184. Academic Press, New York, 1963.

the affinity of biotin for avidin[43] and to characterizing inhibitors of ribulosebisphosphate carboxylase[44] and carboxypeptidase A.[25]

One implication of the theory described in Scheme 1 is that any alteration in structure of the substrate, or of the enzyme, which alters the value of k_{cat} or K_m should have a predictable effect on the binding affinity of an ideal transition state analog inhibitor that perfectly resembles $S^{\ddagger}$. If a change in the structure of the substrate does not affect the rate of the nonenzymatic reaction, then any effect on k_{cat}/K_m should be matched by a change in the affinity of the enzyme for an ideal transition state analog inhibitor. This experimental test of analogy has been passed by inhibitors of papain,[19] elastase,[20] and thermolysin.[45] Alterations in the structure of the enzyme have been examined in the same way. After mutations at the active site, carboxypeptidase A[46] and cytidine deaminase[47] show changes in affinities for transition state analog inhibitors that are closely related to changes in k_{cat}/K_m.

The energetic consequences of stabilizing interactions can be analyzed individually, at least in principle, by deleting one of the interacting groups from either the inhibitor or the enzyme, then examining the thermodynamic consequences of alteration for binding affinity. Kinetic constants such as k_{cat}/K_m are sometimes open to ambiguities of interpretation, because the position of the transition state may vary along the reaction coordinate as alterations are made in the structure of an enzyme or substrate. In contrast, binding affinities of competitive inhibitors offer the advantage of being true dissociation constants that lend themselves to rigorous interpretation. This relationship has been tested for several enzymes by varying the inhibitor[9] and in two cases by varying the structure of the enzyme by site-directed mutagenesis.[46,47]

Examination of enzyme–inhibitor complexes by NMR, revealing states of ionization of both partners in enzyme complexes with transition state analog inhibitors, can provide important indications of the presence of acid–base catalysis as described in the above discussion of analogs of anionic reaction intermediates. States of covalent hydration of inhibitors can be equally revealing, as described in the discussion below of nucleoside deaminases.

[43] N. M. Green, *Biochem. J.* **89**, 585 (1963).
[44] J. V. Schloss, *J. Biol. Chem.* **263**, 4145 (1988).
[45] P. A. Bartlett and C. K. Marlowe, *Biochemistry* **22**, 4618 (1983).
[46] M. A. Phillips, A. P. Kaplan, W. J. Rutter, and P. A. Bartlett, *Biochemistry* **31**, 959 (1992).
[47] A. A. Smith, D. Carlow, R. Wolfenden, and S. A. Short, *Biochemistry* **33**, 6468 (1994).

Slow Binding

Strongly bound inhibitors are often bound slowly: lactate oxidase, for example, binds oxalate with a rate of onset of 80 M^{-1} sec^{-1}.[48] Several caveats should be borne in mind in considering whether these properties are likely to bear any functional relationship to transition state analogy. First, when an inhibitor is bound with high affinity, slow binding (when that is also present) tends to be obvious, simply because the behavior of the inhibitor must be examined at very low concentrations to determine the value of K_i. If, for example, an inhibitor is bound with a K_i value of 10^{-10} M, and the rate constant for formation of EI is 10^7 sec^{-1} M^{-1}, then a slow rate of onset of inhibition becomes obvious in any kinetic investigation intended to determine K_i, carried out over a period of a few minutes. At a concentration of 10^{-10} M, the pseudo-first-order rate constant for the onset of inhibition would be 10^{-3} sec^{-1}. However, if another inhibitor combined with the enzyme at the same rate, but was much less strongly bound ($K_i = 10^{-5}$ M, for example), then its rate of onset of inhibition would be very difficult to determine. For this reason, it remains to be demonstrated in most cases whether "slow" binding is any less common among conventional substrate analog inhibitors than among transition state and multisubstrate analog inhibitors that are much more strongly bound. Second, some transition state analog inhibitors combine rapidly with enzymes, whereas others combine very slowly.[11] If slow binding were an essential, rather than an accidental, feature of transition state analogy, then such variation might not have been expected. Thus, the relationship between transition state affinity and slow binding, if such a relationship exists, is inconsistent, and its origins seem likely to be complex.

Several possible reasons might be advanced to explain slow binding. First, strongly bound inhibitors tend to contain several binding determinants, each of which must be properly engaged for optimal binding. These various interactions might take effect in stages, with some adjustment of the configuration of the active site or the inhibitor that requires the elapse of time. It is also possible that some binding determinants may engage more rapidly than others, resulting in formation of a weak "abortive" complex; this complex must first dissociate before it becomes possible to form the final complex that engages all the appropriate binding determinants. Although rapid weak binding is often followed by slow tight binding, it has seldom if ever been determined whether the rapid weak complex lies on the pathway to the slow tight complex.

[48] S. Ghisla and V. Massey, *J. Biol. Chem.* **250**, 577 (1975).

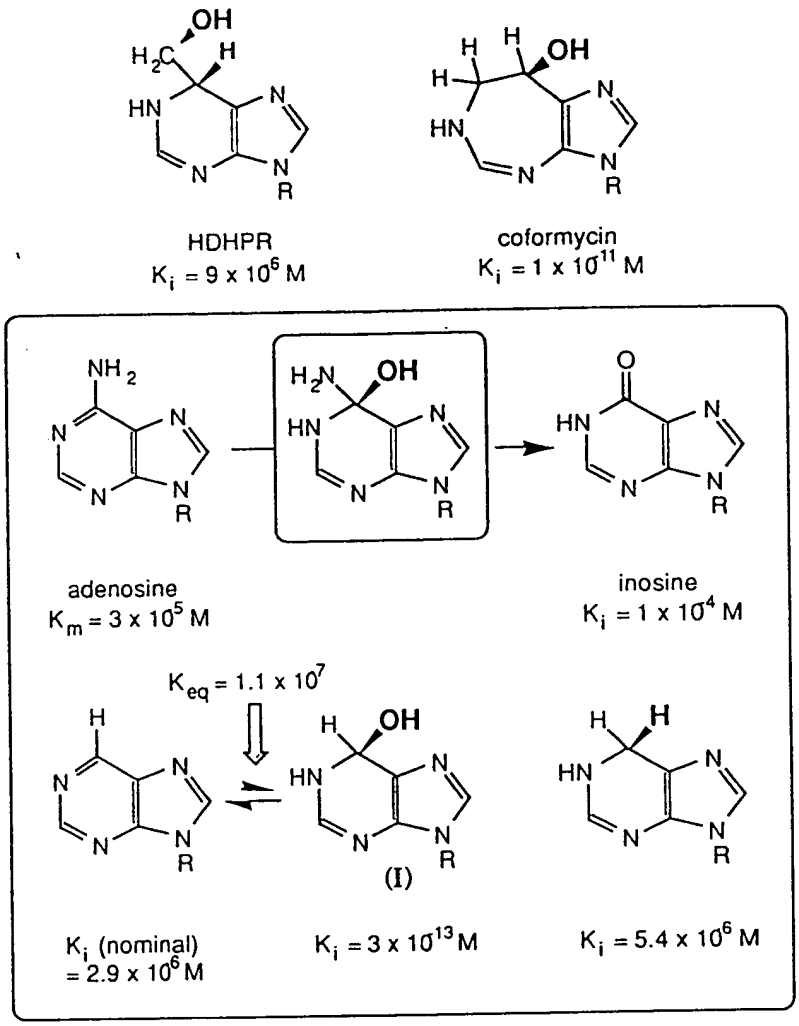
Another explanation for slow binding of transition state analogs may be that the enzyme has not been prepared by natural selection to bind, rapidly, a molecule that resembles the altered substrate in the transition state, although its affinity for that species is extremely high. Ordinarily, the transition state develops from the bound substrate or product, which the enzyme has been prepared by natural selection to bind rapidly, permitting it to act at rates that often approach the limits imposed by encounter between the substrate and enzyme in solution. Subsequent reactions, within the ES complex, may involve topologically complex transformations, including the closing of a lid or flap, to maximize the attractive interactions that so greatly stabilize the altered substrate in the transition state.[49] New crystal structures show that bound transition state analog inhibitors are enveloped so completely that they become almost completely inaccessible to solvent water in the cases of triose-phosphate isomerase,[14] adenosine deaminase,[50] and cytidine deaminase.[51]

Mechanistic Uses of Transition State Analogs: Two Case Histories

Hydrolytic deamination of adenosine, catalyzed by fungal and mammalian enzymes, is strongly inhibited by analogs of an unstable hydrated intermediate formed by 1,6-addition of substrate water approaching from the front side of the adenosine ring as viewed in Scheme 3. Thus, 1,6-hydroxymethyl-1,6-dihydropurine ribonucleoside (HDHPR) and the antibiotics coformycin and 2′-deoxycoformycin are powerful competitive inhibitors. Modeling studies show that the critical hydroxyl group of the hydroxymethyl substituent of the active isomer of HDHPR can be superimposed on the ring hydroxyl group of the natural 8*R*-OH isomer of 2-deoxycoformycin, both compounds being similar in structure to the postulated intermediate in the catalytic process.

In a remarkable display of steric discrimination, adenosine deaminase binds the natural 8*R*-OH isomer of 2-deoxycoformycin more tightly than the synthetic 8*S* isomer by a factor of 10^7.[52] This difference in affinity might arise from strong attraction of the 8*R* isomer by the active site, from steric hindrance of binding of the 8*S* isomer, or from some combination of these effects. In the 8*S* isomer, the critical hydroxyl group projects from the back side of the ring, from which the leaving group is believed to depart during the catalytic process. The extreme lack of specificity of the

[49] R. Wolfenden, *Mol. Cell. Biochem.* **3**, 207 (1974).
[50] D. K. Wilson, F. B. Rudolph, and F. A. Quiocho, *Science* **252**, 1278 (1991).
[51] L. Betts, S. Xiang, S. A. Short, R. Wolfenden, and C. W. Carter, Jr., *J. Mol. Biol.* **235**, 635 (1994).
[52] V. L. Schramm and D. C. Baker, *Biochemistry* **24**, 641 (1985).



SCHEME 3

enzyme with respect to the leaving group (NH_2-, CH_3NH_2-, Cl-, and CH_3O- are similarly reactive) suggests that the active site of the enzyme appears to be "as big as a barn" on the leaving group side, so that steric hindrance is improbable, and the first of these explanations seems most likely to be correct.

Purine ribonucleoside resembles the substrate adenosine except for replacement of the leaving -NH_2 group by hydrogen, and was long considered to be bound by adenosine deaminase as a simple competitive inhibitor with an affinity similar to the apparent affinity of the substrate. That view became untenable when ^{13}C NMR studies revealed that purine ribonucleoside was bound by adenosine deaminase with a change of hybridization

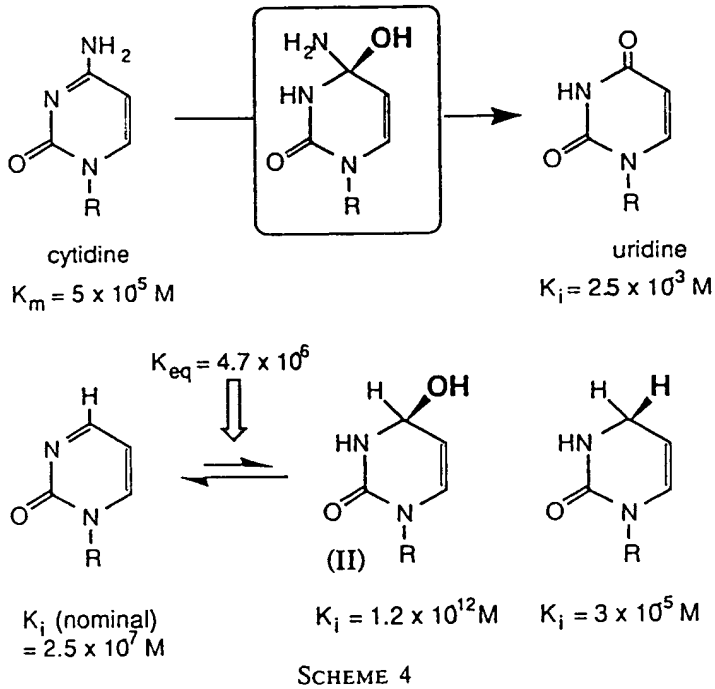
from sp^2 to sp^3 at C-6.[53] The NMR and UV spectra confirmed identification of enzyme-bound purine ribonucleoside as an oxygen adduct, presumably a 1,6-hydrate closely analogous in structure to the 1,6-hydrated intermediate in direct attack by water at the 6 position of adenosine.[5] In this structure, a hydrogen atom occupies the position presumed to be occupied by the leaving $-NH_2$ group in the normal reaction. Because the enzyme is nonspecific for leaving group (-Cl and $-NHCH_3$ are similarly reactive), it is also presumably indifferent to substitution by hydrogen at this position. If the apparent K_i value of purine ribonucleoside is combined with its extremely unfavorable equilibrium constant for hydration in free solution ($K_{eq} = 10^{-7}$), then the true K_i value of the more inhibitory of the two diastereomers of the 1,6-hydrate is found to be in the neighborhood of 3×10^{-13} M.[54]

From the rapid rate of onset of inhibition and the rarity of the hydrate in free solution, it was clear that inhibition normally occurs as a result of purine ribonucleoside binding, followed by hydration at the active site in a mockery of the normal catalytic process.[53] It could further be shown that the equilibrium of hydration is greatly enhanced at the active site of the enzyme, where the effective concentration of substrate water is approximately 10^{10} M.[9]

Cytidine deaminases from bacteria and mammals are strongly inhibited by 3,4,5,6-tetrahydrouridine, structurally analogous to a hypothetical intermediate formed by 3,4-addition of water to the alternate substrate 5,6-dihydrocytidine, shown in Scheme 4. The competitive inhibitors pyrimidin-2-one ribonucleoside [$K_{i(app)} = 3.6 \times 10^{-7}$ M] and 5-fluoropyrimidin-2-one ribonucleoside [$K_{i(app)} = 3.5 \times 10^{-8}$ M] exhibit UV absorption spectra, in their complexes with the enzyme, that are virtually identical with those of the products obtained when hydroxide ion combines with analogs quaternized at N-3.[6] These results indicate that the bound inhibitors are oxygen adducts and provide evidence in favor of binding as a covalent hydrate, not as an enzyme cysteine derivative that had been considered as an alternative possibility. The apparent K_i value of pyrimidin-2-one ribonucleoside as an inhibitor of bacterial cytidine deaminase, combined with its equilibrium constant for covalent hydration in free solution, indicates that $K_i = 1.2 \times 10^{-12}$ M for 3,4-dihydrouridine (the 3,4-hydrate of pyrimidin-2-one ribonucleoside).

Adenosine and cytidine deaminases are nonspecific for the leaving group in substrates, so that they are probably indifferent to replacement of the leaving group by hydrogen in analogs I and II and bind these

[53] L. Kurz and C. Frieden, *Biochemistry* **26**, 8450 (1987).
[54] W. Jones and R. Wolfenden, *J. Am. Chem. Soc.* **108**, 7444 (1986).



SCHEME 4

transition state analogs very tightly. Thus, the hydroxyl group at the sp^3-hybridized carbon atom probably offers one of the few structural features that could be used by either adenosine or cytidine deaminase to distinguish the altered substrate in the transition state for deamination from the substrate in the ground state (Schemes 3 and 4). To assess the contribution of this hydroxyl group to the binding of analogs I and II, we examined the results of replacement by hydrogen. 1,6-Dihydropurine ribonucleoside, prepared photochemically, was found to serve as a simple competitive inhibitor of adenosine deaminase, with a K_i value of 5.4×10^{-6} M. When this value was compared with the K_i value of the 1,6-hydrate of purine ribonucleoside (1.6×10^{-13} M), it became evident that the 6-hydroxyl group of the latter compound contributes -9.8 kcal/mol to the free energy of binding by calf intestinal adenosine deaminase (Scheme 3).[55,56]

Similar experiments on bacterial cytidine deaminase, performed with 3,4-dihydropyrimidin-2-one ribonucleoside ($K_i = 3.0 \times 10^{-5}$ M), showed that the 4-hydroxyl group of 3,4-dihydrouridine contributes -10.1 kcal/mol to the free energy of binding (Scheme 4).[6] Molecular orbital calculations suggest that the geometry and density of electrons are essentially identical at other positions in the hydrogen- and hydroxyl-substituted

[55] W. M. Kati and R. Wolfenden, *Science* **243**, 1591 (1989).
[56] W. M. Kati and R. Wolfenden, *Biochemistry* **28**, 7919 (1989).

ligands, so that these hydroxyl group contributions to binding affinity, approximately −10 kcal/mol, can be considered to result from simple replacement of -OH by -H.

Group Contributions and Role of Solvent Water

When bound by a protein, a ligand must normally be removed, at least in part, from solvent water. To compare the inherent affinities of the desolvated ligands for the active site, it would therefore be of interest to correct for the free energies of their prior removal from solvent water. (Binding also involves removal of the active site from previous contact with solvent water, but this is true in either case and does not contribute to the difference in affinities between the hydroxyl-containing and the hydrogen-containing ligands.) Free energies have now been determined for removal of many compounds of biological interest from solvent water, from their water-to-vapor or water-to-cyclohexane distribution coefficients. To a fair approximation, free energies of solvation of organic compounds are found to vary as an additive function of constituent groups, alcohols being solvated more strongly than the corresponding alkanes by a factor of roughly 10^5.[15] If a hydroxyl-containing ligand is more readily desolvated that the corresponding hydrogen-containing ligands by roughly 7 kcal/mol in free energy, then for both adenosine and cytidine deaminases the contribution of a desolvated hydroxyl group to the binding of a transition state analog inhibitor appears to be approximately −17 kcal/mol.

In arriving at this conclusion, we have assumed that solvent water has been stripped completely from ligands at critical points of contact with the enzyme. That assumption, although it seems plausible for the hydroxylated ligand whose high affinity implies a close fit to the active site, may not be appropriate in the case of the hydrogen-containing ligand. In the latter case a molecule of water may take the place of the missing hydroxyl group. This "trapping" of water would invalidate simple comparison of observed binding affinities as a measure of the contribution of the hydroxyl group to binding affinity. However, if water is trapped in this way, then the stability of the resulting "wet" complex of the hydrogen-containing ligand must presumably be greater than that of any hypothetical "dry" complex of the hydrogen-containing ligand, from which trapped water was absent. Otherwise, a "dry" complex, of the kind needed for direct comparison of binding affinities, would have been formed by the hydrogen-containing ligand. Under these circumstances, the observed difference in binding affinities would be less than the difference in "dry" binding affinities that is needed to determine the contribution of the hydroxyl group to ligand binding.

The meaning of our earlier estimate of the contribution of the critical hydroxyl group to binding, based on the difference in binding affinity between the two ligands, would also be clouded if the conformation of the enzyme were to change and, to a different extent, on binding of the different ligands. The strong affinity observed for the hydroxylated ligand suggests that the native conformation of the enzyme is already well adapted to tight binding of the hydroxyl-containing ligand. The hydrogen-containing ligand, being smaller, should be able to fit into any "native" structure that can accommodate the hydroxylated ligand. It would hardly be surprising, however, if the active site of the enzyme were to show some tendency to collapse around the hydrogen-containing ligand, forming a more compact structure than does the complex of the hydroxyl-containing ligand. Such a change in structure would invalidate simple comparison of binding affinities as a measure of hydroxyl group contribution to binding. If, however, the hydrogen-containing ligand were bound with such a change in conformation, then the stability of the resulting "collapsed" complex would necessarily be greater than that of any complex with the active site in the "native" configuration. Otherwise, the natively configured complex, being more stable, would have been the species actually observed at equilibrium. The contribution of the hydroxyl group to the stability of the complex of the hydroxyl-containing ligand in the native structure would again have been underestimated.

These considerations suggest that if "water trapping" or enzyme distortion accompany formation of the complex of the enzyme with the hydrogen-containing ligand, then either of these effects might be expected to exert a "leveling" influence on the relative affinities observed for the hydroxyl- and hydrogen-containing ligands, leading to underestimation of the contribution of the critical hydroxyl group to binding affinity. Evidently, the contributions of these hydroxyl groups to binding affinities of the desolvated ligands are probably at least as large, and could be larger, than values of approximately -17 kcal/mol suggested by the observed differences in K_i values.

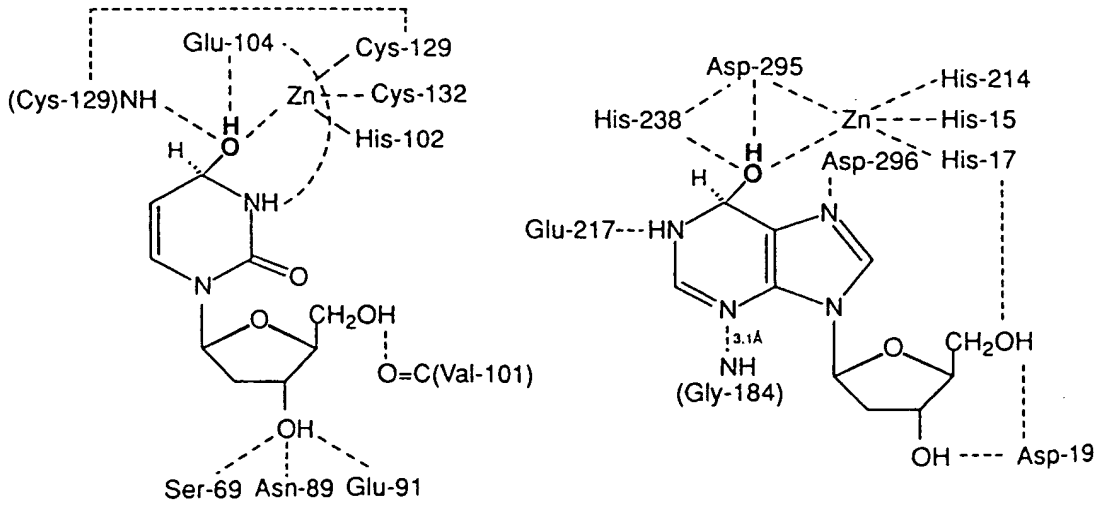
Crystal structures have now been reported for the complexes formed between transition state analogs and adenosine[57] and cytidine[51] deaminases. The results confirm that these inhibitors are bound as the covalent hydrate almost completely removed from contact with solvent water, as in the complex that is formed between 2-phosphoglycolate and triosephosphate isomerase.[14] The implied conformation change, by maximizing the possibility of attractive interactions between the enzyme and substrate in the transition state, may help to answer the conflicting requirements

[57] D. K. Wilson, F. B. Rudolph, and F. A. Quiocho, *Science* 252, 1278 (1991).

of transition state stabilization and rapid access of substrates and egress of products.[49] Several features of the new crystal structures are shown in Scheme 5. The critical hydroxyl group of the inhibitor, on which so much of the catalytic binding enhancement appears to depend, interacts with three groups, including a zinc atom and a carboxylate residue at the active site of the enzyme.

Conclusions

In summary, many of the structural features of a substrate remain unchanged as it passes from the ground state to the transition state. To enhance the rate of a reaction, an enzyme must therefore single out for chemical recognition those few features of a substrate that do change. We have considered the generation of hydrates I and II at the active sites of nucleoside deaminases as analogs of the process by which such enzymes generate intermediates in substrate transformation. In these compounds, a tetrahedrally oriented hydroxyl group is an obvious feature that distinguishes these compounds from the aromatic starting materials. Evidently a few polar interactions involving this group, arising fleetingly in the transition state, are capable of generating a large part of the added binding affinity that is needed to explain the rate enhancement ($\sim 10^{12}$-fold) that an enzyme of this kind produces. Other interactions with the enzyme are obviously important in transition state stabilization and can be analyzed



bacterial cytidine deaminase *mammalian adenosine deaminase*

SCHEME 5

by similar methods, including active site directed and inhibitor directed mutagenesis. Particular mention should be made of the structural results, too extensive for present discussions, of crystallographic studies of other enzyme complexes with transition state and multisubstrate analog inhibitors, especially those of triose-phosphate isomerase,[14] thermolysin,[26] and carboxypeptidase A.[23]

Transition State and Multisubstrate Analogs (Table I)

The list of enzymes and inhibitors in Table I is organized according to EC classification, and an attempt has been made to cite the original reference in each case. A list of this kind is certain to contain errors of omission, for which the authors apologize.

TABLE I
TRANSITION STATE AND MULTISUBSTRATE ANALOGS

EC Number[a]	Enzyme[a]	Inhibitor[b]
1.1.1.1	Alcohol dehydrogenase	NAD^+ adduct[1]
1.1.1.27	L-Lactate dehydrogenase	NAD^+ adduct, oxalate,[2] oxalylethyl-NADH[3]
1.1.1.37	Malate dehydrogenase	NAD^+ adduct[1]
1.1.1.145	3β-Hydroxy-Δ^5-steroid dehydrogenase	4-Aza-4-methyl-5-pregnane-3,20-dione[4]
1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase	Threose-2,4-diphosphate[5]
1.2.7.1	Pyruvate synthase	Thiamine thiazolone and thiamine thiothiazolone pyrophosphates,[6] tetrahydrothiamine pyrophosphate,[7] acetylphosphonate[8]
1.4.1.1	Alanine dehydrogenase	Oxalylethyl-NADH[9]
1.4.1.2	Glutamate dehydrogenase	NAD^+ adduct[10]
1.5.1.3	Dihydrofolate reductase	Methotrexate[11]
1.5.1.X	Deoxyhypusine synthase	*N*-Guanyl-1,7-diaminoheptane[12]
1.11.1.9	Glutathione peroxidase	Mercaptosuccinate[13]
1.13.11.3	Protocatechuate 3,4-dioxygenase	2-Hydroxyisonicotinic acid *N*-oxide[14]
1.13.12.4	Lactate 2-monooxygenase	Oxalate,[15] malonate[16]
1.14.17.1	Dopamine β-monooxygenase	1-(4-Hydroxybenzyl)imidazole-2-thiol[17]
2.1.1.41	24-Sterol *C*-methyltransferase	25-Azacholesterol,[18] 24-(*R,S*)-epiminolanosterol,[19] 24-methyl-25-azacycloartanol[20]
2.1.1.45	Thymidylate synthase	1-(D-2′-Deoxyribofuranosyl)-8-azapurin-2-one 5′-monophosphate,[21] multisubstrate analog[22]
2.1.2.2	Phosphoribosylglycinamide formyltransferase	β-Thioglycinamide ribonucleotide dideazafolate[23]
2.1.3.1	Methylmalonyl-CoA carboxyltransferase	Oxalate[24]
2.1.3.2	Aspartate carbamoyltransferase	Phosphonoacetylaspartate[25]
2.1.3.3	Ornithine carbamoyltransferase	Phosphonoacetylornithine[26,27]
2.3.1.7	Carnitine *O*-acetyltransferase	*O*-[2-(*S*-Coenzyme A)acetyl]carnitine[28]
2.3.1.48	Histone acetyltransferase	*N*-[2-(*S*-Coenzyme A)acetyl]spermidine amide[29]
2.3.1.59	Gentamicin 2′-*N*-acetyltransferase	*N*-[2-(*S*-Coenzyme A)acetyl]gentamicin[30]
2.3.1.X	Succinyl-CoA: tetrahydrodipicolinate *N*-succinyltransferase	2-Hydroxytetrahydropyran-2,6-dicarboxylic acid[31]
2.3.2.2	γ-Glutamyltransferase	Serine–borate complex[32]
2.4.1.1	Glycogen phosphorylase	1,5-Gluconolactone,[33,34] 1-deoxy-α-D-*gluco*-heptulose 2-phosphate[35]
2.4.1.19	Cyclomaltodextrin glucanotransferase	Acarbose[36]
2.4.1.X	α(1 → 2)-Fucosyltransferase	2-*O*-(2-Guanosinophosphonoethyl)-1-*O*-(phenyl)-β-D-galactopyranoside[37]
2.4.2.1	Purine-nucleoside phosphorylase	8-Amino-2′-nordeoxyguanosine, 2′-nordeoxyguanosine diphosphate[38]
2.5.1.1	Geranylgeranyl diphosphate synthase	3-Azageranyl diphosphate[39]
2.5.1.6	Methionine adenosyltransferase	5′-(δ-Methylmethionine)-β,γ-imido-ATP[40]
2.5.1.9	Riboflavin synthase	6,7-Dioxolumazine[41]
2.5.1.16	Spermidine synthase	*S*-Adenosyl-3-thio-1,8-diaminooctane[42]
2.5.1.19	3-Phosphoshikimate 1-carboxyvinyltransferase	Carboxyvallenyl phosphate, (*Z*)-3-fluorophosphoenolpyruvate[43]

2.4.1.1	Glycogen phosphorylase	Serine–borate complex[32]
2.4.1.19	Cyclomaltodextrin glucanotransferase	1,5-Gluconolactone,[33,34] 1-deoxy-α-D-*gluco*-heptulose 2-phosphate[35]
		Acarbose[36]
2.4.1.X	α(1 → 2)-Fucosyltransferase	2-*O*-(2-Guanosinophosphonoethyl)-1-*O*-(phenyl)-β-D-galactopyranoside[37]
2.4.2.1	Purine-nucleoside phosphorylase	8-Amino-2′-nordeoxyguanosine, 2′-nordeoxyguanosine diphosphate[38]
2.5.1.1	Geranylgeranyl diphosphate synthase	3-Azageranyl diphosphate[39]
2.5.1.6	Methionine adenosyltransferase	5′-(δ-Methylmethionine)-β,γ-imido-ATP[40]
2.5.1.9	Riboflavin synthase	6,7-Dioxolumazine[41]
2.5.1.16	Spermidine synthase	*S*-Adenosyl-3-thio-1,8-diaminooctane[42]
2.5.1.19	3-Phosphoshikimate 1-carboxyvinyltransferase	Carboxyallenyl phosphate, (*Z*)-3-fluorophosphoenolpyruvate[43]
2.5.1.21	Farnesyl-diphosphate farnesyltransferase	Ammonium analog of carbocation[44]
2.5.1.22	Spermine synthase	*S*-Adenosyl-1,12-diamino-3-thio-9-azadodecane[45]
2.5.1.26	Alkylglycerone-phosphate synthase	2(3)-Palmitoyl-1,2,3-trihydroxyeicosane 1-phosphate[46]
2.6.1.30	Pyridoxamine–pyruvate transaminase	Pyridoxylalanine[47]
2.7.1.1	Hexokinase	Chromium-ATP[48]
2.7.1.20	Adenosine kinase	Ap_4A[49]
2.7.1.21	Thymidine kinase	Ap_5T[48,50]
2.7.1.40	Pyruvate kinase	Oxalate[51]
2.7.3.2	Creatine kinase	Nitrate[52]
2.7.3.3	Arginine kinase	Nitrate[53,54]
2.7.4.3	Adenylate kinase	Ap_5A[55]
2.7.9.2	Pyruvate, water dikinase	Oxalate[56]
2.7.10.X	p70 S6 kinase	Rapamycin[57]
2.8.3.5	3-Oxoacid CoA-transferase	Succinic monohydroxamic acid[58]
3.1.1.1	Carboxylesterase	Benzil,[59] ethylphenyl glyoxalate[60]
3.1.1.4	Phospholipase A_2	1-Hexadecylthio-2-hexadecanoylamino-1,2-dideoxy-*sn*-glycero-3-phosphocholine, 1-hexadecylthio-1-deoxy-2-hexadecylphosphono-*sn*-glycero-3-phosphocholine,[61] 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol[62]
3.1.1.7	Acetylcholinesterase	Boronate,[63] fluoroketone[64,65]
3.1.1.8	Cholinesterase	Diphenylboric acid[66]
3.1.1.13	Sterol esterase	Phenyl-*n*-butylborinic acid,[67] chlorodifluoroacetophenone[68]
3.1.1.34	Lipoprotein lipase	Phenyl-*n*-butylborinic acid[69]
3.1.1.59	Juvenile-hormone esterase	Fluoroketones[70]
3.1.3.1	Alkaline phosphatase	Vanadate[71]
3.1.3.2	Acid phosphatase	Tungstate, molybdate[72]
3.1.6.1	Arylsulfatase	Sulfite[73]

(*continued*)

TABLE I (*continued*)

EC Number[a]	Enzyme[a]	Inhibitor[b]
3.1.27.5	Pancreatic ribonuclease	Uridine-vanadate[74]
3.2.1.1	α-Amylase	D-*malto*-Bionolactone[75]
3.2.1.10	*O*-Glycosyl glycosidase	Acarbose,[76] bis(hydroxymethyl)dihydroxypyrrolidine[77]
3.2.1.17	Lysozyme	Lactone, 2-acetamido-2-deoxyglucoseen[78]
3.2.1.18	Exo-α-sialidase	2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid[79]
3.2.1.21	β-Glucosidase	1-Aminoglucoside,[80] gluconolactone[81]
3.2.1.22	Galactosidase	1-Aminogalactoside,[82] galactal[83]
3.2.1.23	β-Galactosidase	1-Aminogalactoside,[82] galactal[83]
3.2.1.45	Glucosylceramidase	Glucosylamine[84]
3.2.1.48	Sucrose α-glucosidase	Castanospermine,[85] 2,6-Dideoxy-2,6-imino-7-*O*-(β-D-glucopyranosyl)-D-*glycero*-L-*gulo*-heptitol,[86]
3.2.1.52	β-*N*-Acetylhexosaminidase	NAG-lactone[87]
3.2.1.55	α-*N*-Arabinofuranosidase	L-Arabino-1,4-lactone[88]
3.2.2.4	AMP nucleosidase	Formycin 5′-phosphate[89]
3.2.2.X	Uracil-DNA-glycosylase	6-(*p*-*n*-Octylanilino)uracil[90]
3.4.11.1	Leucyl aminopeptidase	Aminoaldehydes,[91] bestatin,[92] amastatin,[93] aminohydroxamates[94]
3.4.15.1	Peptidyl-dipeptidase A	Captopril,[95] enalapril,[96] fluoroketone,[97] aminoalcohol,[98] 2-mercaptoacetyl dipeptides,[99] ketodimethyl peptide,[100] aminoketodimethyl peptides[101]
3.4.17.1	Carboxypeptidase A	Benzylsuccinate,[102] L-2-phosphoryloxy-3-phenylpropionic acid,[103] dipeptide phosphoramidates,[104] 2-benzyl-3-formylpropionate, 2-mercaptoacetyl dipeptides,[76] 3-phosphonopropionic acid,[105] (2-carboxy-3-phenylpropyl)methylsulfoximine and -sulfodiimine,[106] phosphonotetrapeptides[107]
3.4.17.2	Carboxypeptidase B	Benzylsuccinate[108]
3.4.17.3	Lysine carboxypeptidase	2-Mercaptomethyl-3-guanidinoethyl thiopropionate,[109] phosphono dipeptide[110]
3.4.19.3	Pyroglutamyl-peptidase I	Oxoprolinal[111]
3.4.21.1	Chymotrypsin	Aldehyde,[112] 1-acetamido-2-phenylethaneboronic acid,[113] peptidyl fluoromethyl ketones[114]
3.4.21.4	Trypsin	Peptide and nonpeptide borates[115]
3.4.21.36	Pancreatic elastase	Peptidyl fluoromethylketones,[116] acetyl-Pro-Ala-Pro-alaninal[117]
3.4.21.62	Subtilisin	Benzeneboronic acid[118]
3.4.22.2	Papain	Acetyl-Phe-glycinal,[119] *N*-acetyl-1-phenylalanylaminoacetonitrile[120]
3.4.22.3	Ficain	Acetyl-Phe-glycinal[121]
3.4.23.1	Pepsin A	Pepstatin,[122] methylpepstatin and statone,[123] fluoroketone,[124] phosphinic acid dipeptide[125]

3.4.21.4	Trypsin	methyl ketones[114]
3.4.21.36	Pancreatic elastase	Peptide and nonpeptide borates[115]
3.4.21.62	Subtilisin	Peptidyl fluoromethylketones,[116] acetyl-Pro-Ala-Pro-alaninal[117]
		Benzeneboronic acid[118]
3.4.22.2	Papain	Acetyl-Phe-glycinal,[119] *N*-acetyl-1-phenylalanylaminoacetonitrile[120]
3.4.22.3	Ficain	Acetyl-Phe-glycinal[121]
3.4.23.1	Pepsin A	Pepstatin,[122] methylpepstatin and statone,[123] fluoroketone,[124] phosphinic acid dipeptide[125]
3.4.23.15	Renin	Statine,[126] difluorostatine and difluorostatone,[127] reduced peptide,[128] dihydroxyethylene peptide analogs[129]
3.4.23.X	HIV protease	Hydroxyethylene peptide isostere[130]
3.4.24.3	Microbial collagenase	Isoamylphosphonyl peptide,[131] cinnamoyl-Phe-(CO)Gly-Pro-Pro ketone,[132] phosphonamide[133]
3.4.24.11	Neprilysin	*N*-Carboxymethyl peptides,[137] 2-mercaptoacetyl dipeptides[76]
3.4.24.27	Thermolysin	*N*-Carboxymethyl dipeptides,[134] phosphonamidate,[135] dipeptides,[76] hydroxamates[83,136]
3.5.1.1	Asparaginase	Aspartate semialdehyde[138]
3.5.1.4	Amidase	Acetaldehyde–ammonia[139]
3.5.2.3	Dihydroorotase	Borocarbamylethyl aspartate[140]
3.5.2.6	β-Lactamase	Arylmethyl phosphonate methyl ester[141]
3.5.4.3	Guanine deaminase	(1,2,6)-Thiadiazine-1,1-dioxides[142]
3.5.4.4	Adenosine deaminase	1,6-Dihydro-6-hydroxymethylpurine ribonucleoside,[143] coformycin,[144] 1,6-dihydroinosine[145]
3.5.4.5	Cytidine deaminase	Tetrahydrouridine,[146] phosphapyrimidine,[147] 1,3-diazepin-2-ol ribonucleoside,[148] 3,4-dihydrouridine[148a]
3.5.4.6	AMP deaminase	Coformycin 5′-phosphate[149]
3.5.4.12	dCMP deaminase	Tetrahydrouridine 5′-phosphate[150]
3.11.1.1	Phosphonoacetaldehyde hydrolase	Phosphite + acetaldehyde[151]
	Cholesterol 5,6-oxide hydrolase	5,6-Iminocholestanol[152]
4.1.1.3	Oxaloacetate decarboxylase	Oxalate[153]
4.1.1.4	Acetoacetate decarboxylase	Acetopyruvate,[154] acetoacetone[155]
4.1.1.23	Orotidine-5′-phosphate decarboxylase	1-(5′-Phospho-D-ribosyl)barbituric acid[156]
4.1.1.39	Ribulose-bisphosphate carboxylase	Carboxyribitol diphosphate,[157] carboxyarabinitol diphosphate[158]
4.1.2.13	Fructose-bisphosphate aldolase	Phosphoglycolohydroxamate[159]
4.1.3.1	Isocitrate lyase	3-nitropropionate[160]
4.1.3.7	Citrate (*si*)-synthase	Carboxymethyl-CoA and oxaloacetate,[161] *S*-acetonyl-CoA[162]
4.1.99.1	Tryptophanase	2,3-Dihydrotryptophan[159]
4.1.99.4	1-Aminocyclopropane-1-carboxylate deaminase	1-Aminocyclopropane phosphonate[163]

(*continued*)

TABLE I (*continued*)

EC Number[a]	Enzyme[a]	Inhibitor[b]
4.2.1.2	Fumarate hydratase	3-Nitro-2-hydroxypropionate,[164] *S*-2,3-dicarboxyaziridine[165]
4.2.1.3	Aconitate hydratase	Nitro analogs of citrate and isocitrate[166]
4.2.1.17	Enoyl-CoA hydratase	Acetoacetyl-CoA[167]
4.2.1.20	Tryptophan synthase	2,3-Dihydrotryptophan[168]
4.3.1.1	Aspartate ammonia-lyase	3-Nitro-2-aminopropionate[169]
4.3.1.5	Phenylalanine ammonia-lyase	L-2-Aminooxy-3-phenylpropionic acid[170]
4.3.2.2	Adenylosuccinate lyase	N^6-(L-2-Carboxyethyl-2-nitroethyl)-AMP[171]
4.4.1.5	Lactoylglutathione lyase	3-Hydroxy-2-methyl-4*H*-pyran-4-one, isoascorbate,[172] isopropyltropolon[173]
5.1.1.1	Alanine racemase	1-Aminocyclopropane phosphonate[164]
5.1.1.4	Proline racemase	Pyrrole,[174] pyrroline 2-carboxylates[175]
5.1.1.7	Diaminopimelate epimerase	3-Chlorodiaminopimelic acid[176]
5.3.1.1	Triose-phosphate isomerase	2-Phosphoglycolate,[177] 2-phosphoglycolohydroxamate[178]
5.3.1.6	Ribose-5-phosphate isomerase	4-Phosphoerythronate[179]
5.3.1.9	Glucose-6-phosphate isomerase	5-Phosphoarabinonate[180]
5.3.1.13	Arabinose-5-phosphate isomerase	4-Phosphoerythronate[181]
5.3.3.1	Steroid Δ-isomerase	17β-D-hydroequinalin[182]
5.3.3.2	Isopentenyl-diphosphate Δ-isomerase	2-(Dimethylamino)ethyl pyrophosphate[183]
5.4.2.2	Phosphoglucomutase	α-D-glucose 1-phosphate vanadate[184]
5.4.99.5	Chorismate mutase	Oxabicyclo[3.3.1]nonene,[185] 2-aza-2,3-dihydrosqualene[186]
6.3.1	Aminoacyl-tRNA ligases	Aminoalkyl adenylates,[187] aminophosphonyl adenylates[188]
6.3.1.2	Glutamate–ammonia ligase	Phosphinotricin,[189] methionine sulfoximine phosphate[190]
6.3.2.2	Glutamate–cysteine ligase	Buthionine-sulfoximine phosphate[191]
6.4.1.1	Pyruvate carboxylase	Oxalate[192]

[a] Classification and nomenclature of enzymes based on recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, published in 1992 (E. C. Webb, ed., Academic Press).

[b] *Key to references:* (*1*) J. Everse, E. C. Zoll, L. Kahan, and N. O. Kaplan, *Biorg. Chem.* **1,** 207 (1971); (*2*) W. B. Novoa, A. D. Winer, A. J. Glaid, and G. W. Schwert, *J. Biol. Chem.* **234,** 1143 (1959); (*3*) H. Kapmeyer, G. Pfleiderer, and W. E. Trommer, *Biochemistry* **15,** 5024 (1976); (*4*) P. J. Bertics, C. F. Edman, and H. J. Karavolas, *J. Biol. Chem.* **259,** 107 (1984); (*5*) A. L. Fluharty and C. E. Ballou, *J. Biol. Chem.* **234,** 2517 (1958); (*6*) T. A. Gutowski and G. E. Lienhard, *J. Biol. Chem.* **251,** 2863 (1976); (*7*) P. N. Lowe, F. J. Leeper, and R. N. Perham, *Biochemistry* **22,** 150 (1983); (*8*) R. Kluger, *J. Am. Chem. Soc.* **99,** 4504 (1977); (*9*) H. Kapmeyer, G. Pfleiderer, and W. E. Trommer, *Biochemistry* **15,** 5024 (1976); (*10*) J. Everse, E. C. Zoll, L. Kahan, and N. O. Kaplan, *Bioorg. Chem.* **259,** 1043 (1984); (*11*) W. C. Werkheiser, *J. Biol. Chem.*

236, 888 (1961); (*12*) J. Jakus, E. C. Wolff, M. H. Park, and J. E. Folk, *J. Biol. Chem.* **268,** 13151 (1993); (*13*) J. Chaudiere, E. C. Wilhemsen, and A. L. Tappel, *J. Biol. Chem.* **259,** 1043 (1984); (*14*) S. W. May, C. D. Oldham, P. W. Mueller, S. R. Padgette, and A. L. Sowell, *J. Biol. Chem.* **257,** 12746 (1982); (*15*) S. Ghisla and V. Massey, *J. Biol. Chem.* **250,** 577 (1975); (*16*) S. Ghisla and V. Massey, *J. Biol. Chem.* **252,** 6729 (1977); (*17*) L. I. Kruse, W. E. DeWolf, Jr., P. A. Chambers, and P. J. Googhart, *Biochemistry* **25,** 7271 (1986); (*18*) A. C. Oehlschlager, R. H. Angus, A. M. Pierce, H. D. Pierce, Jr., and R. Srinivasan, *Biochemistry* **23,** 3582 (1984); (*19*) W. D. Nes, G. G. Jansen, R. A. Norton, [illegible] and L. Johsson, *Biochem. Biophys. Res. Commun.* **177,** 566 (1991); (*20*) A. Rahier,

(4) P. J. Bertics, C. F. Edman, and H. J. Karavolas, *J. Biol. Chem.* **259,** 107 (1984); (5) A. L. Fluharty and C. E. Ballou, *J. Biol. Chem.* **234,** 2517 (1958); (6) T. A. Gutowski and G. E. Lienhard, *J. Biol. Chem.* **251,** 2863 (1976); (7) P. N. Lowe, F. J. Leeper, and R. N. Perham, *Biochemistry* **22,** 150 (1983); (8) R. Kluger, *J. Am. Chem. Soc.* **99,** 4504 (1977); (9) H. Kapmeyer, G. Pfleiderer, and W. E. Trommer, *Biochemistry* **15,** 5024 (1976); (10) J. Everse, E. C. Zoll, L. Kahan, and N. O. Kaplan, *Bioorg. Chem.* **259,** 1043 (1984); (11) W. C. Werkheiser, *J. Biol. Chem.*

[11]

236, 888 (1961); (12) J. Jakus, E. C. Wolff, M. H. Park, and J. E. Folk, *J. Biol. Chem.* **268,** 13151 (1993); (13) J. Chaudiere, E. C. Wilhemsen, and A. L. Tappel, *J. Biol. Chem.* **259,** 1043 (1984); (14) S. W. May, C. D. Oldham, P. W. Mueller, S. R. Padgette, and A. L. Sowell, *J. Biol. Chem.* **257,** 12746 (1982); (15) S. Ghisla and V. Massey, *J. Biol. Chem.* **250,** 577 (1975); (16) S. Ghisla and V. Massey, *J. Biol. Chem.* **252,** 6729 (1977); (17) L. I. Kruse, W. E. DeWolf, Jr., P. A. Chambers, and P. J. Googhart, *Biochemistry* **25,** 7271 (1986); (18) A. C. Oehlschlager, R. H. Angus, A. M. Pierce, H. D. Pierce, Jr., and R. Srinivasan, *Biochemistry* **23,** 3582 (1984); (19) W. D. Nes, G. G. Jansen, R. A. Norton, M. Kalinowska, F. G. Crumley, B. Tal, A. Bergenstrahle, and L. Johsson, *Biochem. Biophys. Res. Commun.* **177,** 566 (1991); (20) A. Rahier, D. Benveniste, and F. Schubert, *J. Am. Chem. Soc.* **103,** 2408 (1981); (21) T. I. Kalman and D. Goldman, *Biochem. Biophys. Res. Commun.* **102,** 682 (1981); (22) A. Srinivasan, V. Armarnath, A. D. Broom, F. C. Zou, and Y.-C. Cheng, *J. Med. Chem.* **27,** 1710 (1984); (23) J. Inglese, R. A. Blatchy, and S. J. Benkovic, *J. Med. Chem.* **32,** 937 (1989); (24) D. Northrop and H. G. Wood, *J. Biol. Chem.* **244,** 5820 (1969); (25) K. D. Collins and G. R. Stark, *J. Biol. Chem.* **246,** 6599 (1971); (26) M. Mori, K. Aoyagi, M. Tatibana, T. Ishikawa, and H. Ishii, *Biochem. Biophys. Res. Commun.* **76,** 900 (1977); (27) N. J. Hoogenraad, *Arch. Biochem. Biophys.* **188,** 137 (1978); (28) J. F. A. Chase and P. K. Tubbs, *Biochem. J.* **111,** 225 (1969); (29) P. M. Cullis, R. Wolfenden, L. S. Cousens, and B. M. Alberts, *J. Biol. Chem.* **257,** 12165 (1982); (30) J. W. Williams and D. B. Northrop, *J. Antibiot.* **32,** 1147 (1979); (31) D. A. Berges, W. E. DeWolf, Jr., G. L. Dunn, D. J. Newman, S. J. Schmidt, J. J. Taggart, and C. Gilvarg, *J. Biol. Chem.* **261,** 6160 (1986); (32) S. S. Tate and A. Meister, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 4806 (1978); (33) J. I. Tu, G. R. Jacobson, and D. J. Graves, *Biochemistry* **10,** 1229 (1971); (34) A. M. Gold, E. Legrand, and G. R. Sanchez, *J. Biol. Chem.* **246,** 5700 (1971); (35) H. W. Klein, M. J. Im, and D. Palm, *Eur. J. Biochem.* **157,** 107 (1986); (36) A. Nakamura, K. Haga, and K. Yamane, *Biochemistry* **32,** 6624 (1993); (37) M. M. Palcic, L. D. Heerze, O. P. Srivastava, and O. Hindsgaul, *J. Biol. Chem.* **264,** 17174 (1989); (38) J. M. Stein, J. D. Stoeckler, S.-Y. Li, R. L. Tolman, M. MacCoss, A. Chen, J. D. Karkas, W. T. Ashton, and R. E. Parks, Jr., *Biochem. Pharmacol.* **36,** 1237 (1987); (39) H. Sagami, T. Korenaga, K. Ogura, A. Steiger, H.-J. Pyun, and R. M. Coates, *Arch. Biochem. Biophys.* **297,** 314 (1992); (40) F. Kappler and A. Hampton, *J. Med. Chem.* **33,** 2545 (1990); (41) S. S. Al-Hassan, R. J. Kuilick, D. B. Livingstone, C. J. Suckling, and H. C. S. Wood, *J. Chem. Soc., Perkins Trans. 1* 2645 (1980); (42) K. C. Tang, A. E. Pegg, and J. K. Coward, *Biochem. Biophys. Res. Commun.* **96,** 1371 (1980); (43) M. C. Walker, J. E. Ream, R. D. Sammons, E. W. Logusch, M. H. O'Leary, R. L. Sommerville, and J. A. Sikorski, *BioMed. Chem. Lett.* **1,** 683 (1991); (44) R. M. Sandifer, M. D. Thompson, R. G. Gaughan, and C. D. Poulter, *J. Am. Chem. Soc.* **104,** 7376 (1982); (45) P. A. Woster, A. Y. Black, K. D. Duff, J. K. Coward, and A. E. Pegg, *J. Med. Chem.* **32,** 1300 (1989); (46) S. Hixson and R. Wolfenden, *Biochem. Biophys. Res. Commun.* **101,** 1064 (1981); (47) W. B. Dempsy and E. E. Snell, *Biochemistry* **2,** 1414 (1963); (48) K. D. Danenberg and W. W. Cleland, *Biochemistry* **14,** 28 (1975); (49) R. Bone, Y. C. Cheng, and R. Wolfenden, *J. Biol. Chem.* **261,** 5731 (1986); (50) S. Ikeda and D. H. Ives, *J. Biol. Chem.* **23,** 12659 (1985); (51) G. H. Reed and S. D. Morgan, *Biochemistry* **13,** 3537 (1974); (52) E. J. Milner-White and D. C. Watts, *Biochem. J.* **122,** 727 (1971); (53) D. H. Buttlaire and M. Cohn, *J. Biol. Chem.* **249,** 5733 (1974); (54) D. H. Buttlaire and M. Cohn, *J. Biol. Chem.* **249,** 5741 (1974); (55) G. E. Lienhard and I. I. Secemski, *J. Biol. Chem.* **248,** 1121 (1973); (56) S. Narindrasorasak and W. A. Bridger, *Can. J. Biochem.* **56,** 816 (1978); (57) C. J. Kuo, J. Chung, D. F. Fiorentino, W. M. Flanagan, J. Blenis, and G. R. Crabtree, *Nature (London)* **358,** 70 (1992); (58) C. M. Pickart and W. P. Jencks, *J. Biol. Chem.* **254,** 9120 (1979); (59) M. C. Berndt, J. de Jersey, and B. Zerner, *J. Am. Chem. Soc.* **99,** 8332 (1977); (60) M. C. Berndt, J. de Jersey, and B. Zerner, *J. Am. Chem. Soc.* **99,** 8334

(*continued*)

TABLE I (*continued*)

(1977); (*61*) L. Yu and E. A. Dennis, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 9325 (1991); (*62*) M. K. Jain, W. Tao, J. Rogers, C. Arenson, H. Eibl, and B.-Z. Yu, *Biochemistry* **30,** 10256 (1991); (*63*) K. A. Koehler and G. P. Hess, *Biochemistry* **13,** 5345 (1974); (*64*) U. Brodbeck, K. Schweikert, R. Gentinetta, and M. Rottenberg, *Biochem. Biophys. Acta* **567,** 357 (1979); (*65*) M. H. Bleb, J. P. Svaren, and R. H. Abeles, *Biochemistry* **24,** 1813 (1985); (*66*) C. W. Garner, G. W. Little, and J. W. Pelley, *Biochim. Biophys. Acta* **790,** 91 (1984); (*67*) L. D. Sutton, J. S. Stout, L. Hosie, P. S. Spencer, and D. M. Quinn, *Biochem. Biophys. Res. Commun.* **134,** 386 (1986); (*68*) J. Sohl, L. D. Sutton, D. J. Burton, and D. M. Quinn, *Biochem. Biophys. Res. Commun.* **151,** 554 (1988); (*69*) L. D. Sutton, J. S. Stout, L. Hosie, P. S. Spencer, and D. M. Quinn, *Biochem. Biophys. Res. Commun.* **134,** 386 (1986); (*70*) B. D. Hammock, K. D. Wing, J. McLaughlin, V. M. Lovell, and T. C. Sparks, *Pestic. Biochem. Physiol.* **17,** 76 (1982); (*71*) V. Lopez, T. Stevens, and R. N. Lindquist, *Arch. Biochem. Biophys.* **175,** 31 (1976); (*72*) R. L. Van Etten, P. P. Waymack, and D. M. Rehkop, *J. Am. Chem. Soc.* **96,** 6782 (1974); (*73*) A. B. Roy, *Biochem. J.* **55,** 653 (1955); (*74*) R. N. Lindquist, J. L. Lynn, Jr., and G. E. Lienhard, *J. Am. Chem. Soc.* **95,** 8762 (1973); (*75*) E. Laszlo, J. Hollo, A. Hoschke, and G. Sarosi, *Carbohydr. Res.* **61,** 387 (1978); (*76*) D. Schmidt, W. Frommer, B. Junge, L. Muller, W. Wingender, E. Truscheit, and D. Schafer, *Naturwissenschaften* **64,** 535 (1977); (*77*) M. Schindler and N. Sharon, *J. Biol. Chem.* **251,** 4330 (1976); (*78*) P. J. Card and W. D. Hitz, *J. Org. Chem.* **50,** 891 (1985); (*79*) C. A. Miller, P. Wand, and M. Flashner, *Biochem. Biophys. Res. Commun.* **83,** 1479 (1978); (*80*) H. L. Lai and B. Axelrod, *Biochem. Biophys. Res. Commun.* **54,** 463 (1973); (*81*) C. D. Santos and W. R. Terra, *Biochim. Biophys. Acta* **831,** 179 (1985); (*82*) H. L. Lai and B. Axelrod, *Biochem. Biophys. Res. Commun.* **54,** 463 (1973); (*83*) D. F. Wentworth and R. Wolfenden, *Biochemistry* **13,** 4715 (1974); (*84*) K. M. Osiecki-Newman, D. Fabbro, G. Legler, R. J. Desnick, and G. A. Grabowski, *Biochim. Biophys. Acta* **915,** 87 (1987); (*85*) C. Danzin and A. Ehrhardt, *Arch. Biochem. Biophys.* **257,** 472 (1987); (*86*) B. L. Rhinehart, K. M. Robinson, P. S. Liu, A. J. Payne, M. E. Wheatley, and S. R. Wagner, *J. Pharmacol. Exp. Ther.* **241,** 915 (1987); (*87*) Y. C. Lee, *Biochem. Biophys. Res. Commun.* **35,** 161 (1969); (*88*) A. H. Fielding, M. L. Sinnott, M. A. Kelly, and D. Widows, *J. Chem. Soc., Perkin Trans. 1* 1013 (1981); (*89*) W. E. de Wolf, F. A. Fullin, and V. L. Schramm, *J. Biol. Chem.* **254,** 10868 (1979); (*90*) F. Focher, A. Verri, S. Spadari, R. Manservigi, J. Gambino, and G. E. Wright, *Biochem. J.* **292,** 883 (1993); (*91*) L. Andersson, T. C. Isley, and R. Wolfenden, *Biochemistry* **21,** 4177 (1982); (*92*) H. Umezawa, H. Aoyagi, H. Suda, M. Hamada, and T. Takeuchi, *J. Antibiot.* **29,** 97 (1976); (*93*) D. H. Rich, B. J. Moon, and S. Harbeson, *J. Med. Chem.* **27,** 417 (1984); (*94*) W. W. C. Chan, P. Dennis, W. Demmer, and K. Brand, *J. Biol. Chem.* **257,** 7955 (1982); (*95*) D. W. Cushman, H. S. Cheung, E. F. Sabo, and M. A. Ondetti, *Biochemistry* **16,** 5484 (1977); (*96*) A. A. Patchett *et al., Nature* (*London*) **288,** 280 (1980); A. A. Patchett and E. H. Cordes, *Adv. Enzymol. Relat. Areas Mol. Biol.* **57,** 1 (1985); (*97*) U. Brodbeck, K. Schweikert, R. Gentinetta, and M. Rottenberg, *Biochim. Biophys. Acta* **567,** 357 (1979); (*98*) E. M. Gordon, J. D. Godfrey, J. Pluscel, D. von Langen, and S. Natarajan, *Biochem. Biophys. Res. Commun.* **126,** 419 (1985); (99) B. Holmquist and B. L. Vallee, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 6216 (1979); (*100*) E. M. Gordon, S. Natarajan, J. Pluscec, H. N. Weller, J. D. Godfrey, M. B. Rom, E. F. Sabo, J. Engebrecht, and D. W. Cushman, *Biochem. Biophys. Res. Commun.* **124,** 148 (1984); (*101*) E. M. Gordon, S. Natarajan, J. Pluscec, H. N. Weller, J. D. Godfrey, M. B. Rom, E. F. Sabo, J. Engebrecht, and D. W. Cushman, *Biochem. Biophys. Res. Commun.* **126,** 419 (1985); (*102*) L. D. Byers and R. Wolfenden, *J. Biol. Chem.* **247,** 606 (1972); (*103*) D. W. Cushman, H. S. Cheung, E. F. Sabo, and M. A. Ondetti, *Biochemistry* **16,** 5484 (1977); (*104*) N. E. Jacobsen and P. A. Bartlett, *J. Am. Chem. Soc.* **103,** 654 (1981); (*105*) D. Grobelny, U. B. Goli, and R. E. Galardy, *Biochem. J.* **232,** 15 (1985); (*106*) W. L. Mock and J.-T. Tsay, *J. Am. Chem. Soc.* **111,** 4467 (1989); (*107*) J. E. Hanson, A. P. Kaplan, and P. A. Bartlett, *Biochemistry* **28,** 6294 (1989); (*108*) D. J. McKay and T. H. Plummer, *Biochemistry* **17,** 401 (1978); (*109*) T. H. Plummer and T. J. Ryan, *Biochem. Biophys. Res. Commun.* **98,** 4481 (1981); (*110*) K. Yamauchi, S. Ohtsuki, and M. Kinoshita, *Biochim. Biophys. Acta* **827,** 275 (1985); (*111*) T. C. Friedman, T. K. Kline, and S. Wilk, *Biochemistry* **24,** 3907 (1985); (*112*) E. J. Breaux and M. L. Bender, *FEBS Lett.* **56,** 81 (1975); (*113*) K. A. Koehler and G. E. Lienhard, *Biochemistry* **10,** 2477 (1971); (*114*) B. Imperiali and R. H. Abeles, *Biochemistry* **25,** 3760 (1986); (*115*) W. W. Bachovchin, W. Y. L. Wong, S. Farr-Jons, A. B. Shenvi, and C. A. Kettner, *Biochemistry* **27,** 12839

(*102*) L. D. Byers and R. Wolfenden, *J. Biol. Chem.* **247**, 606 (1972); (*103*) D. W. Cushman, H. S. Cheung, E. F. Sabo, and M. A. Ondetti, *Biochemistry* **16**, 5484 (1977); (*104*) N. E. Jacobsen and P. A. Bartlett, *J. Am. Chem. Soc.* **103**, 654 (1981); (*105*) D. Grobelny, U. B. Goli, and R. E. Galardy, *Biochem. J.* **232**, 15 (1985); (*106*) W. L. Mock and J.-T. Tsay, *J. Am. Chem. Soc.* **111**, 4467 (1989); (*107*) J. E. Hanson, A. P. Kaplan, and P. A. Bartlett, *Biochemistry* **28**, 6294 (1989); (*108*) D. J. McKay and T. H. Plummer, *Biochemistry* **17**, 401 (1978); (*109*) T. H. Plummer and T. J. Ryan, *Biochem. Biophys. Res. Commun.* **98**, 4481 (1981); (*110*) K. Yamauchi, S. Ohtsuki, and M. Kinoshita, *Biochim. Biophys. Acta* **827**, 275 (1985); (*111*) T. C. Friedman, T. K. Kline, and S. Wilk, *Biochemistry* **24**, 3907 (1985); (*112*) E. J. Breaux and M. L. Bender, *FEBS Lett.* **56**, 81 (1975); (*113*) K. A. Koehler and G. E. Lienhard, *Biochemistry* **10**, 2477 (1971); (*114*) B. Imperiali and R. H. Abeles, *Biochemistry* **25**, 3760 (1986); (*115*) W. W. Bachovchin, W. Y. L. Wong, S. Farr-Jons, A. B. Shenvi, and C. A. Kettner, *Biochemistry* **27**, 12839 (1988); (*116*) B. Imperiali and R. H. Abeles, *Biochemistry* **25**, 3760 (1986); (*117*) R. C. Thompson, *Biochemistry* **12**, 47 (1973); (*118*) R. N. Lindquist and C. Terry, *Arch. Biochem. Biophys.* **160**, 135 (1974); (*119*) J. O. Westerik and R. Wolfenden, *J. Biol. Chem.* **247**, 8195 (1971); (*120*) T.-C. Liang and R. H. Abeles, *Arch. Biochem. Biophys.* **252**, 626 (1987); (*121*) C. A. Lewis, Jr., Ph.D. Thesis, University of North Carolina, Chapel Hill (1976); (*122*) H. Umezawa, T. Aoyagi, H. Morishima, M. Matzusaki, H. Hamada, and T. Takeuchi, *J. Antibot.* **23**, 259 (1970); (*123*) D. H. Rich, M. S. Bernatowicz, N. S. Agarwal, M. Kawai, and F. G. Salituro, *Biochemistry* **24**, 3165 (1985); (*124*) M. H. Gelb, J. P. Svaren, and R. H. Abeles, *Biochemistry* **24**, 1813 (1985); (*125*) P. H. Bartlett and W. B. Keyer, *J. Am. Chem. Soc.* **106**, 4282 (1984); (*126*) J. Boger, L. S. Payne, D. S. Perlow, N. S. Lohr, M. Poe, E. H. Blaine, E. H. Ulm, T. W. Schorn, B. I. LaMont, T.-Y. Lin, M. Kawai, D. H. Rich, and D. F. Veber, *J. Med. Chem.* **28**, 1779 (1985); (*127*) S. Thaisrivongs, D. T. Pals, W. Kati, S. R. Turner, L. M. Thomasco, and W. Watt, *J. Med. Chem.* **29**, 2080 (1986); (*128*) M. Szelke, B. Leckie, A. Hallett, D. M. Jones, J. Sueiras, B. Atrash, and A. F. Lever, *Nature* (*London*) **299**, 555 (1982); (*129*) J. R. Luly, N. BaMaung, J. Soderquist, A. K. L. Fung, H. Stein, H. D. Kleinert, P. A. Marcotte, D. A. Egan, B. Bopp, I. Merits, G. Bolis, J. Greer, T. J. Perun, and J. J. Plattner, *J. Med. Chem.* **31**, 2264 (1988); (*130*) G. B. Dreyer, B. W. Metcalf, T. A. Tomaszek, T. J. Carr, A. C. Chandler, III, L. Hyland, S. A. Fakhoury, V. W. Magaard, M. L. Moore, J. E. Strickler, C. Debouck, and T. D. Meek, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9752 (1989); (*131*) R. E. Galardy and D. Grobelny, *Biochemistry* **22**, 4556 (1983); (*132*) D. Grobelny, C. Teater, and R. E. Galardy, *Biochem. Biophys. Res. Commun.* **159**, 426 (1989); (*133*) V. Dive, A. Yiotakis, A. Nicolaou, and F. Toma, *Eur. J. Biochem.* **191**, 685 (1990); (*134*) A. L. Maycock, D. M. De Sousa, L. G. Payne, J. ten Broeke, M. T. Wu, and A. A. Patchett, *Biochem. Biophys. Res. Commun.* **102**, 963 (1981); (*135*) P. A. Bartlett and C. K. Marlowe, *Biochemistry* **22**, 4618 (1983); P. A. Bartlett and C. K. Marlowe, *Biochemistry* **26**, 8553 (1987); (*136*) N. Nishino and J. C. Powers, *Biochemistry* **13**, 2846 (1978); (*137*) S. Almenoff and M. Orlowski, *Biochemistry* **22**, 590 (1983); (*138*) J. O. Westerik and R. Wolfenden, *J. Biol. Chem.* **249**, 6351 (1974); (*139*) J. D. Findlater and B. A. Orsi, *FEBS Lett.* **35**, 109 (1973); (*140*) D. H. Kinder, S. K. Frank, and M. M. Ames, *J. Med. Chem.* **33**, 819 (1990); (*141*) J. Rahil and R. F. Pratt, *Biochemistry* **31**, 5869 (1992); (*142*) R. B. Meyer and E. B. Skibo, *J. Med. Chem.* **22**, 944 (1979); (*143*) B. Evans and R. Wolfenden, *J. Am. Chem. Soc.* **92**, 4751 (1970); (*144*) T. Sawa, Y. Fukagawa, I. Homma, T. Takeuchi, and H. Umezama, *J. Antibiot.* **204**, 227 (1967); (*145*) W. Jones, L. C. Kurz and R. Wolfenden, *Biochemistry* **28**, 1242 (1989); (*146*) R. M. Cohen and R. Wolfenden, *J. Biol. Chem.* **246**, 7561 (1971); (*147*) G. W. Ashley and P. A. Bartlett, *J. Biol. Chem.* **259**, 13621 (1984); (*148*) V. E. Marquez, P. S. Liu, J. A. Kelley, J. S. Driscoll, and J. J. McCormack, *J. Med. Chem.* **23**, 713 (1980); (*148a*) L. Frick, C. Yang, V. E. Marquez, and R. Wolfenden, *Biochemistry* **28**, 9423 (1989); (*149*) C. Frieden, H. R. Gilbert, W. H. Miller, and R. L. Miller, *Biochem. Biophys. Res. Commun.* **91**, 278 (1979); (*150*) F. Maley and G. F. Maley, *Arch. Biochem. Biophys.* **144**, 723 (1971); (*151*) J. M. Lanauze, H. Rosenberg, and D. C. Shaw, *Biochim. Biophys. Acta* **212**, 332 (1970); (*152*) N. T. Nashed, D. P. Michaud, W. Levin, and D. M. Jerina, *Arch. Biochem. Biophys.* **241**, 149 (1985); (*153*) A. Schmitt, I. Bottke, and G. Siebert, *Hoppe-Seyler's Z. Physiol. Chem.* **347**, 18 (1966); (*154*) W. Tagaki, J. P. Guthrie, and F. H. Westheimer, *Biochemistry* **7**, 905 (1968); (*155*) I. Fridovich, *J. Biol. Chem.* **243**, 1043 (1968); (*156*) B. W. Potvin, H. J. Stern, S. R. May, G. R. Lam, and R. S. Krooth, *Biochem. Pharmacol.* **27**, 655 (1978); (*157*) M.

(*continued*)

TABLE I (*continued*)

Wishnick, M. D. Lane, and M. C. Scrutton, *J. Biol. Chem.* **245,** 4939 (1970); (*158*) J. Pierce, N. E. Tolbert, and R. Barker, *Biochemistry* **19,** 934 (1980); J. V. Schloss, *J. Biol. Chem.* **263,** 4145 (1988); (*159*) K. D. Collins, *J. Biol. Chem.* **249,** 136 (1974); (*160*) J. V. Schloss and W. W. Cleland, *Biochemistry* **21,** 4420 (1982); (*161*) E. Bayer, B. Bauer, and H. Eggerer, *Eur. J. Biochem.* **120,** 155 (1981); (*162*) P. Rubenstein and R. Dryer, *J. Biol. Chem.* **255,** 7858 (1980); L. C. Kurz, S. Shah, B. C. Crane, L. J. Donald, H. W. Duckworth, and G. R. Drysdale, *Biochemistry* **31,** 7899 (1992); (*163*) M. D. Erion and C. T. Walsh, *Biochemistry* **26,** 3417 (1987); (*164*) D. J. T. Porter and H. J. Bright, *J. Biol. Chem.* **255,** 4772 (1980); (*165*) J. Greenhut, H. Umezawa, and F. B. Rudolph, *J. Biol. Chem.* **260,** 6684 (1985); (*166*) J. V. Schloss, D. J. T. Porter, H. J. Bright, and W. W. Cleland, *Biochemistry* **19,** 2358 (1980); (*167*) R. M. Waterson and R. L. Hill, *J. Biol. Chem.* **247,** 5258 (1972); (*168*) R. S. Phillips, E. W. Miles, and L. A. Cohen, *Biochemistry* **23,** 6228 (1984); (*169*) D. J. T. Porter and H. J. Bright, *J. Biol. Chem.* **255,** 4772 (1980); (*170*) K. R. Hanson, *Arch. Biochem. Biophys.* **211,** 575 (1981); (*171*) D. J. T. Porter, N. G. Rudie, and H. J. Bright, *Arch. Biochem. Biophys.* **225,** 157 (1983); (*172*) K. T. Douglas and I. N. Nadvi, *FEBS Lett.* **106,** 393 (1979); (*173*) J. F. Barnard and J. F. Honek, *Biochem. Biophys. Res. Commun.* **165,** 118 (1989); (*174*) G. J. Cardinale and R. H. Abeles, *Biochemistry* **7,** 3970 (1968); (*175*) M. V. Keenan and W. L. Alworth, *Biochem. Biophys. Res. Commun.* **57,** 500 (1974); (*176*) R. J. Baumann, E. H. Bohme, J. S. Wiseman, M. Vaal, and J. S. Nicols, *Antimicrob. Agents Chemother.* **32,** 1119 (1988); (*177*) R. Wolfenden, *Biochemistry* **9,** 3404 (1970); (*178*) K. D. Collins, *J. Biol. Chem.* **249,** 136 (1974); (*179*) W. W. Woodruff and R. Wolfenden, *J. Biol. Chem.* **254,** 5866 (1979); (*180*) J. M. Chirgwin and E. A. Noltmann, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **32,** 667 (1973); (*181*) E. C. Bigham, C. E. Gragg, W. R. Hall, J. E. Kelsey, W. R. Mallory, D. C. Richardson, C. Benedict, and P. H. Ray, *J. Med. Chem.* **27,** 717 (1984); (*182*) S. Wang, F. S. Kawahara, and D. Talalay, *J. Biol. Chem.* **238,** 576 (1963); (*183*) J. E. Reardon and R. H. Abeles, *Biochemistry* **25,** 5609 (1986); M. Muehlbacher and C. D. Poulter, *Biochemistry* **27,** 7315 (1988); (*184*) W. J. Ray and J. M. Puvathingal, *Biochemistry* **29,** 2790 (1990); (*185*) P. A. Bartlett and C. R. Johnson, *J. Am. Chem. Soc.* **107,** 7792 (1985); (*186*) A. Duriatti, P. Bouvier-Nave, P. Benveniste, F. Schuber, L. Delprino, G. Balliano, and L. Cattel, *Biochem. Pharmacol.* **34,** 2765 (1985); (*187*) D. Cassio, F. Le Moine, J. P. Waller, E. Sandrin, and R. A. Boissonas, *Biochemistry* **6,** 827 (1967); (*188*) A. I. Biryukov, B. Kh. Ishmuratov, and R. M. Khomutov, *FEBS Lett.* **91,** 249 (1978); (*189*) E. W. Logusch, D. M. Walker, J. F. McDonald, and J. E. Franz, *Biochemistry* **28,** 3043 (1989); (*190*) W. B. Rowe, R. A. Ronzio, and A. Meister, *Biochemistry* **8,** 2674 (1969); (*191*) O. W. Griffith, *J. Biol. Chem.* **257,** 13704 (1982); (*192*) A. S. Mildvan, M. C. Scrutton, and M. F. Utter, *J. Biol. Chem.* **241,** 3488 (1966).